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TELOMERASE ACTIVATION AND ITS CLINICAL IMPLICATIONS IN HUMAN THYROID TUMORS

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Telomerase activation and its clinical implications in human thyroid tumors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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路漫漫其修远兮，吾将上下而求索

屈原《离骚》

The road ahead will be long and our climb will be steep.

To my family

ABSTRACT

Telomerase is a ribonucleoprotein enzyme. The catalytic and rate-limiting component of telomerase is referred to as telomerase reverse transcriptase (TERT) that plays an important role in telomere maintenance, cell immortalization and malignant transformation. Due to its aberrant expression and critical role in cancer development and progression, TERT has been suggested as a promising molecular marker for cancer diagnosis and prognosis. Thyroid cancer is the most common endocrine cancer. The diagnosis and treatment for thyroid cancer has been extensively studied; however, the molecular mechanisms underlying the roles of TERT in thyroid cancer are incompletely understood. The aim of this thesis was to explore the regulation, biological role and clinical significance of TERT in thyroid cancer.

Somatic mutations of the *TERT* promoter, a genetic event for telomerase activation, were first described in malignant melanoma and have then been identified in various types of cancer during the last two years. The *TERT* promoter mutations were detected in follicular thyroid cell- derived thyroid carcinomas but not in parafollicular C cell-derived medullary thyroid carcinoma (MTC); the presence of the mutation was associated with shorter telomeres, poor outcome and aggressive clinical features. *TERT* promoter mutation was also found in follicular thyroid adenoma with uncertain malignant potential, which indicates that it is an early genetic event in thyroid carcinogenesis.

Despite the absence of *TERT* promoter mutations, telomerase activation including *TERT* expression and telomerase activity was detected in more than half of MTCs, and associated with aggressive disease behavior and poor outcome. These results suggest that TERT can serve as a useful prognostic marker for MTC. It was further revealed that *TERT* copy number alterations, *TERT* promoter methylation, different *TERT* RNA splicing variants and other cancer-related proteins contributed to the up-regulation of *TERT* expression in MTC. Besides telomerase activation, alternative lengthening of telomere (ALT) was identified as another mechanism for telomere length maintenance in MTC.

The known oncogenic *RET* and *RAS* mutations were also detected in MTCs; however, no association was found between these two genetic events and clinical features. The tyrosine kinase inhibitor vandetanib decreased MTC cell viability in *in vitro* experiments, but proteins regulated in response to the *RET* pathway inhibition need to be further clarified.

In summary, this thesis provides further insights into the molecular mechanisms for telomerase activation and aberrant TERT induction, and the roles of TERT/telomerase in human thyroid carcinogenesis. More importantly, the findings underline the clinical importance of TERT/telomerase assessment in thyroid cancer.

LIST OF SCIENTIFIC PAPERS

I. Liu T[#], **Wang N**[#], Cao J, Sofiadis A, Dinets A, Zedenius J, Larsson C[*] and Xu D[*]

The age- and shorter telomere-dependent *TERT* promoter mutation in follicular thyroid cell-derived carcinomas.
Oncogene, 2014, 33(42):4978-4984

II. **Wang N**[#], Liu T[#], Sofiadis A, Juhlin CC, Zedenius J, Höög A, Larsson C[*] and Xu D[*]

TERT promoter mutation as an early genetic event activating telomerase in follicular thyroid adenoma (FTA) and atypical FTA.
Cancer, 2014, 120(19): 2965-2979

III. **Wang N**[*], Xu D, Sofiadis A, Höög A, Vukojevic V, Bäckdahl M, Zedenius J, Larsson C[*]

Telomerase-dependent and independent telomere maintenance and its clinical implications in medullary thyroid carcinoma
The Journal of Clinical Endocrinology and Metabolism, 2014, 99(8), E1571-1579

IV. **Wang N**[*], Kjellin H, Sofiadis A, Fotouhi O, Juhlin CC, Bäckdahl M, Zedenius J, Xu D, Lehtiö J and Larsson C

Genetic background and protein expression profiles in relation to telomerase activation in medullary thyroid carcinoma
Submitted

V. **Wang N**[*], Kjellin H, Orre LM, Sofiadis A, Juhlin CC, Bäckdahl M, Zedenius J, Lehtiö J and Larsson C

Protein expression profiles of medullary thyroid carcinoma – relation to *RET* mutational status and vandetanib treatment.
Manuscript

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1 Ge Z, Li W, **Wang N**, Liu C, Zhu Q, Björkholm M, Gruber A, Xu D

Chromatin remodeling: recruitment of histone demethylase RBP2 by Mad 1 for transcriptional repression of a Myc target gene, telomerase reverse transcriptase.
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3 Liu T, Brown TC, Juhlin CC, Andreasson A, **Wang N**, Bäckdahl M, Healy JM, Prasad ML, Korah R, Carling T, Xu D, Larsson C

The activating *TERT* promoter mutation C228T is recurrent in subsets of adrenal tumors.
Endocrine- Related Cancer. 2014, 21(3):427-434

4 Xie H, Liu T, **Wang N**, Björnhagen V, Höög A, Larsson C, Liu WO, Xu D

TERT promoter mutations and gene amplification: promoting *TERT* expression in Merkel cell carcinoma.
Oncotarget. 2014, 5(20): 10048-10057.

CONTENTS

LIST OF ABBREVIATIONS

AFTA	Atypical follicular thyroid adenoma
AKT(PKB)	Protein kinase B
ALT	Alternative lengthening of telomere
AP-2	Activating enhancer-binding protein-2
APB	ALT-associated promyelocytic leukemia protein nuclear bodies
APS	Adenosine-5´-phophosulfate
ATC	Anaplastic thyroid carcinoma
ATG	Translation starting site
CCD	Charged coupled device
cDNA	Complementary DNA
ddNTP	Dideoxynucleotide
DIG	Digoxigenin
E6-AP	E6 associated protein
ERK	Extracellular signal-regulated kinases
ETS	E26 transformation specific
FFPE	Formalin fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
FNA	Fine needle aspiration
FTA	Follicular thyroid adenoma
FTC	Follicular thyroid carcinoma
GDNF	Glial-derived neurotrophic factor
GFR	GDNF-family receptor
HIF-1	Hypoxia-inducible factor-1
HMGA2	High-mobility group A2
HPV	Human papillomavirus
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinases
MCAF1	MBD1-containing chromatin-associated factor 1
MEN 2	Multiple endocrine neoplasia type 2

MS	Mass spectrometry
MTC	Medullary thyroid carcinoma
NFAT	Nuclear factor of activated T cell
NFX1	Nuclear transcription factor, X-box binding 1
NUPR1	Nuclear protein 1
P1TX1	Paired-like homeodomain transcription factor 1
PAX8	Paired box 8
PCR	Polymerase Chain Reaction
PDTC	Poorly differentiated thyroid carcinoma
PI3K	Phosphatidylinositide 3-kinases
PKC	Protein kinase C
PML	Promyelocytic leukemia
PPARγ	Peroxisome proliferator activated receptor γ
PTC	Papillary thyroid carcinoma
T3	Triiodothyronine
T4	Thyronine
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TRF	Terminal restriction fragment
TRH	Thyrotropin-releasing hormone
TSG	Tumor suppressor gene
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptors
VGF	Nerve growth factor inducible
WT1	Wilms´ tumor 1

1 INTRODUCTION

1.1 CANCER AND ITS MOLECULAR GENETIC BACKGROUND

1.1.1 Multi-step molecular genetic development of cancer

Cancer arises from cells in normal human tissues that grow out of control and may invade adjacent and distant tissues. Cancer is a disease of genes, and the genetic instability that is characteristic of cancer drives the tumor development. Normal cells divide and die under the strict control of complicated molecular mechanisms, and they are able to mature into cells with well-differentiated phenotypes and specific cellular functions. Normal cells are also capable of eliminating or repairing genetic errors or mistakes that occur as they divide; otherwise they will launch the cell death program. However, cancer cells are able to keep the genetic errors or damages without dying and transfer them to their daughter cells. The accumulation of these molecular changes will prompt them to replicate uncontrolled, to acquire more and more genetic abnormalities, to break down the normal tissue, and to invade surrounding or distant tissues. An example of cancer development and progression is illustrated in **Figure 1**. The initiation and progression of cancer follow a Darwinian type of evolution, that generally takes decades of time and involves multiple genetic and epigenetic changes. Today we know that six biological capabilities are acquired during cancer development to conquer the barriers of cell death: evading of growth suppressors, sustaining of proliferative signaling, resistance to cell death, induction of angiogenesis, enabling of replicative immortality and activation of mechanisms for invasion and metastasis [1, 2].

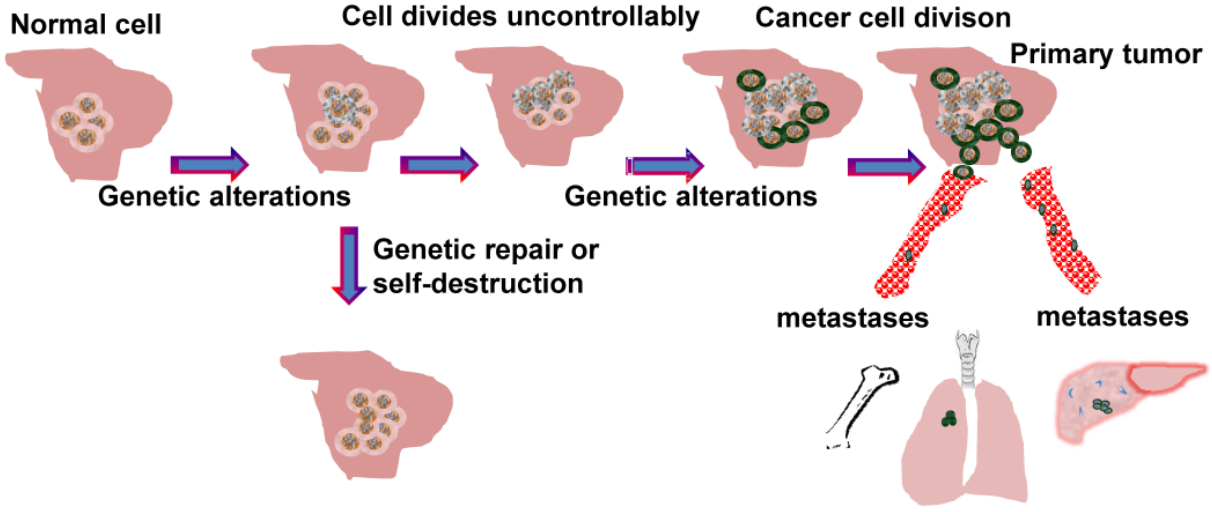

Figure 1. The model of multi-step development of cancer.

1.1.2 Genetic changes in cancer

In the late nineteenth and early twentieth centuries, David von Hansemann and Theodor Boveri proposed that cancer is caused by abnormalities of hereditary materials, which was supported by the discovery of DNA and mutagens of cancer. During the last century, a large

body of cancer research has demonstrated that alterations of genes controlling cell proliferation and survival will lead to cancer. As shown in **Figure 2**, there are many types of genetic alterations for example gene mutations and numerical and structural alterations of chromosomes or chromosomal loci (loss or gain, chromosome rearrangement). The genetic abnormality is the consequence of one or combinations of these alterations.

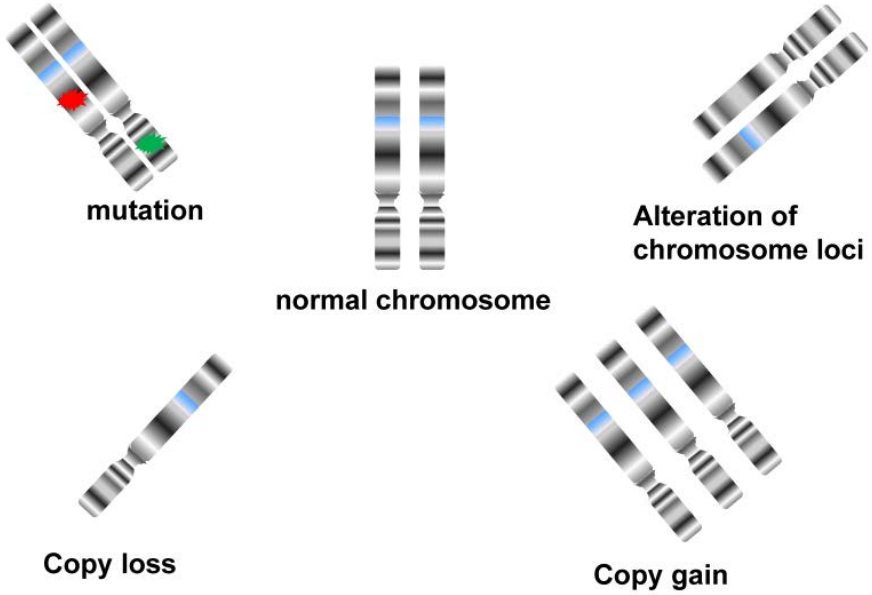

Figure 2. **Examples of genetic alterations in human cancer.**

1.1.2.1 Gene mutations

Mutations may result from exposure to different mutagens or by chance during cell division. Based on the DNA alteration and whether it alters the genetic code or not, mutations are categorized into different classes. Base substitutions where a single base is replaced are frequent, and can induce altered peptide sequence (non-synonymous mutation) affecting a single amino acid (missense mutation) or giving a shorter protein from a premature stop-codon (non-sense mutation). A single base replacement that does not change the affected amino acid is called synonymous mutation. In addition, the mutation may involve addition or loss of base pairs where one or more nucleotides are either deleted from the gene (deletion mutation), or inserted into the sequence (insert mutation) and which may shift the reading frame (frame-shift mutation). Besides these common types of mutations, dynamic mutations of unstable genetic sequences also occur. Depending on the cell type in which the mutation occurs, they are classified as constitutional or somatic mutations. Both of them may cause cancer in the human body.

1.1.2.1.1 Constitutional mutations

In the case of constitutional mutations, the heritable information is altered by mutated gene alleles, which will be transmitted by germ cells from parents to offspring. This type of mutation, which is present in all the cells of the body, is either inherited from a parent or has occurred *de novo* and can be passed from generation to generation. Around 5% to 10% of

human cancers are induced by a constitutional mutation, which is referred to as familial cancer. Multiple endocrine neoplasia type 2 (MEN 2) with familiar occurrence of medullary thyroid carcinoma (MTC) and other endocrine tumors is a good example of inherited cancer predisposition caused by a constitutional mutation.

1.1.2.1.2 Somatic mutations

Somatic mutations occur in single somatic cells, which will affect the phenotype of the cell. They may be acquired after exposure to mutagens in the environment or surrounding cellular microenvironment or they may occur by coincidence. Somatic mutations can be passed to the descendant cells within the same tissue, but not to the offspring. Most human cancers have been shown to carry cancer-related somatic mutations, and these mutations play a central role in the development of cancer.

1.1.2.2 Chromosomal aberrations

As early as in 1914, chromosomes abnormality in cancer cells were observed by light microscopy [3]; hence they are referred to as large-scaled alterations. Similar to the small-scaled alterations in cancer cells, chromosomal abnormalities may occur on the somatic or constitutional level. Alterations involving the structure and number of chromosomes are the main categories of chromosomal aberrations in human cancer.

1.1.2.2.1 Structural abnormalities

Eukaryotic chromosomes consist of three DNA sequence elements: DNA replication elements, telomeres and centromeres. During the cell cycles, the chromosome structure displays quite different appearances. The prometaphase and metaphase chromosome has two chromatids, while in the interphase the chromosome shows a single chromatid and DNA double helix structure.

Structural aberrations can be induced by DNA damage or DNA recombination. Chromosome breaks that occur in a single chromosome following DNA damage can lead to insertion, deletion, inversion, duplication, ring chromosome or gene rearrangements; breaks in two chromosomes trigger chromosomal translocations. The abnormal chromosome structure may affect genes that are crucial for cancer development, leading to activation, over-expression or inactivation. .

1.1.2.2.2 Numerical abnormalities

The normal chromosomal composition is a euploid karyotype, however, aneuploidy is frequently observed in cancer cells. The acquiring of extra copies of chromosomes or the loss of a chromosome copy can benefit the cancer cells and prompt its uncontrolled growth.

1.1.3 Epigenetic changes in cancer

The term epigenetics refer to heritable changes in cells that do not affect the underlying DNA sequences. Epigenetic mechanisms are crucial for gene expression in human cells and are

essential for the normal cell development and differentiation. Thus, epigenetic studies are expected to contribute to the understanding of differences in morphology and function between cells that carry the same DNA sequence. The epigenetic modifications can either activate or inhibit gene expression. Researches over the past decades have highlighted the important role of epigenetic dysregulations in the initiation and progression of human cancer. DNA methylation, histone modification, and RNA-mediated gene expression regulation are three main epigenetic systems involved in gene regulation.

1.1.3.1 DNA methylation

DNA methylation was proposed to regulate gene expression during cell development and differentiation in 1975 [4, 5]. Since then, plenty of research has proved that DNA methylation is essential in regulation of gene expression in normal cells and cancer [6-13]. Generally, DNA methylation occurs in cytosine guanine dinucleotides, which are distributed in clusters commonly known as CpG islands. The methylation can induce covalent binding of a methyl group to the C-5 position of a CpG dinucleotide. The CpG-rich areas span the regulatory region of many genes at the 5´ end [14]. In normal cells, these CpG islands are commonly un-methylated or have a very low level of methylation [15]. By contrast in most tumor cells altered methylation levels may be observed in promoter regions [16, 17].

Altered methylation involves DNA hypomethylation and hypermethylation, and both are known to contribute to cancer cell development [18-20]. CpG islands at the gene promoter region are seldomly methylated under normal conditions, but are frequently found to be hypermethylated or hypomethylated in different types of cancer. Hypermethylation in the promoter region of tumor suppressor genes (TSGs) is the major epigenetic event in cancers known today. Inactivation of TSGs by promoter hypermethylation is illustrated in **Figure 3**. In the classical situation this will inactive TSGs through reduced expression with consequences for various cell regulations. Therefore, methylation of CpG islands was considered as one of the hallmarks of cancer [21, 22]. The increasingly noted hypomethylation at repetitive sequences, coding sequences and introns associated with tumor progression is less clarified [23, 24]. Loss of methylation may facilitate for deletions and translocations to occur and thus increase the frequency of chromosomal instability [18, 25]; it can also disrupt the genomic imprinting and thereby increase the risk of cancer [26].

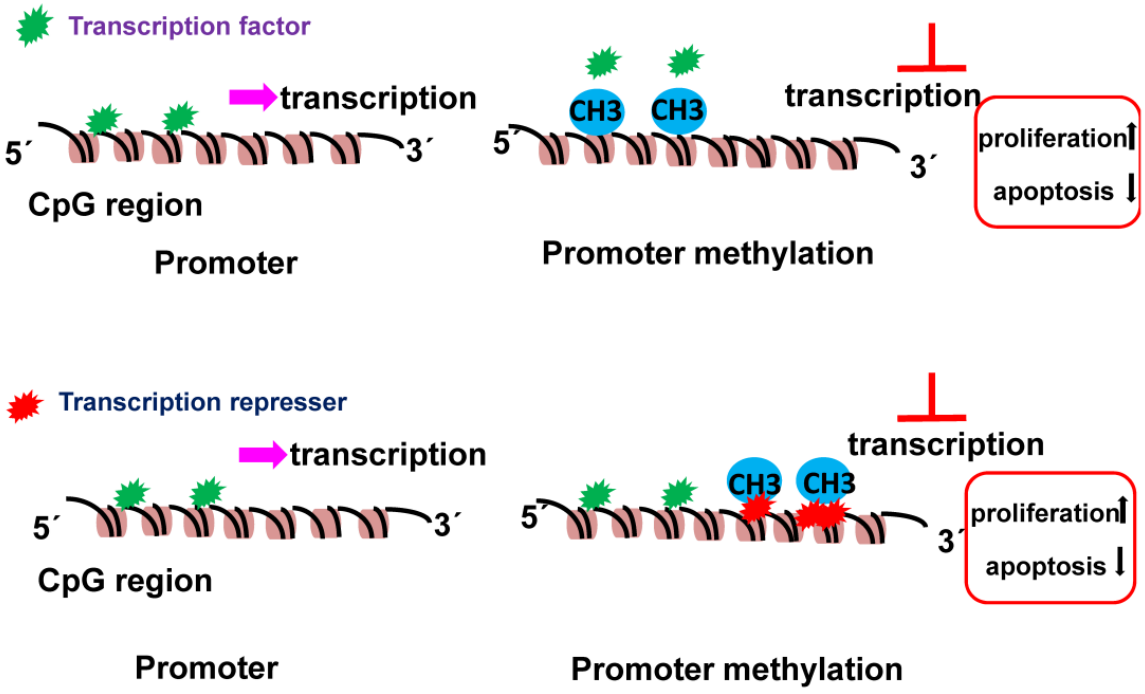

Figure 3. Tumor suppressor gene suppression by DNA hypermethylation.

1.1.3.2 Histone modification

In eukaryotic cell nuclei, the DNA molecule is organized in nucleosomes by a highly alkaline protein called histone. The five known histone family proteins are the main functional proteins in chromatin. Histone 1 and histone 5 are linker proteins, and histone 2, 3 and 4 are core assembly proteins of the nucleosome. Within the chromatin, DNA is twisted around the histones; hence, histones work as spools in chromatin. Histones can also be involved in gene regulation including gene activation and repression. The amino acids on the long histone tail can be covalently modified, thereby inducing interactions with DNA and with other nuclear proteins. Several different types of histone modifications are known such as acetylation, methylation, ubiquitination, and phosphorylation. Methylation and acetylation play a role in DNA replication, transcription regulation and DNA repair. The methylation of certain histone residues are related to different biological processes. Histone modifications are known to contribute to the development of cancer in different organs [27-29].

1.1.3.3 RNA-mediated gene regulation

During the last two decades, the increasing knowledge of RNA molecules has expanded the understanding of gene regulation in cancer dramatically. This is especially evident for the research following the discovery of microRNA in 1993 [30]. MicroRNAs are short, small, around 22-nucleotide non-coding RNAs. Its special sequence can imperfectly complement with the 3´ un-translated region (3´-UTR) of target mRNAs, thereby inducing translational repression [31]. Research on microRNA expression in cancer has revealed that expression differs between tumor and normal tissue, and the down-regulation of certain microRNAs has important roles in gene regulation in cancer [32-34]. The regulation of microRNAs may

involve the microRNAs processing machinery or epigenetic mechanisms. The translational silencing by non-coding RNAs has therefore been considered as one type of epigenetic mechanism for gene regulation [35-37].

1.1.4 Important genes involved in cancer

Human cancer commonly exhibits multiple chromosomal alterations and mutations. Three main types of genes play a crucial role in tumorigenesis and contribute to cancer formation and development: oncogenes, tumor suppressor genes and DNA repair genes.

1.1.4.1 Proto-oncogenes

Proto-oncogenes are growth-controlling genes in normal human cells, which were discovered by retrovirologists in the 1970s. They usually participate in the receiving, transducing and processing of growth or proliferation signals to cells. A proto-oncogene may turn into a cancer-causing oncogene if it is altered in certain ways, such as by genetic alterations affecting the protein structure or expression. Examples of such underlying genetic alterations are chromosomal rearrangements giving new regulatory elements or fusion genes, and activating mutations. These changes will release signals to stimulate the cells to proliferate out of control. Mutated *RET* and *RAS* genes are well known oncogenes in human cancers.

1.1.4.2 Tumor suppressor genes

Tumor suppressor genes (TSGs) are involved in the government of dynamic cell proliferation coupled with proto-oncogenes, and regulate cell proliferation via different mechanisms. Inactivation of TSGs will disrupt the control of cell proliferation and has been observed more frequently in cancer than activation of oncogenes. The normal function of a TSG may be lost by several different mechanisms: loss of heterozygosity (LOH), genetic mutations and epigenetic silencing are the most commonly observed. Inactivation of the TSG *TP53* is seen in the majority of human cancers.

1.1.4.3 DNA repair genes

The normal systems for genome maintenance provide another important barrier to cancer, and deficiencies in these systems contribute to cancer development. Nucleotide repair, mismatch repair, homologous recombination, end joining and telomere maintenance are important biological mechanisms for genome stability.

1.2 THE HUMAN THYROID GLAND AND THYROID TUMORS

1.2.1 The thyroid gland and its physiological function

The thyroid gland is located in front of and surrounding the trachea in the neck. As shown in **Figure 4**, the thyroid gland consists of two lobes connected by the isthmus in the middle. The cellular composition includes a majority of follicular cells (thyrocytes), parafollicular calcitonin-producing C-cells, endothelial cells, fibroblasts and lymphocytes [38].

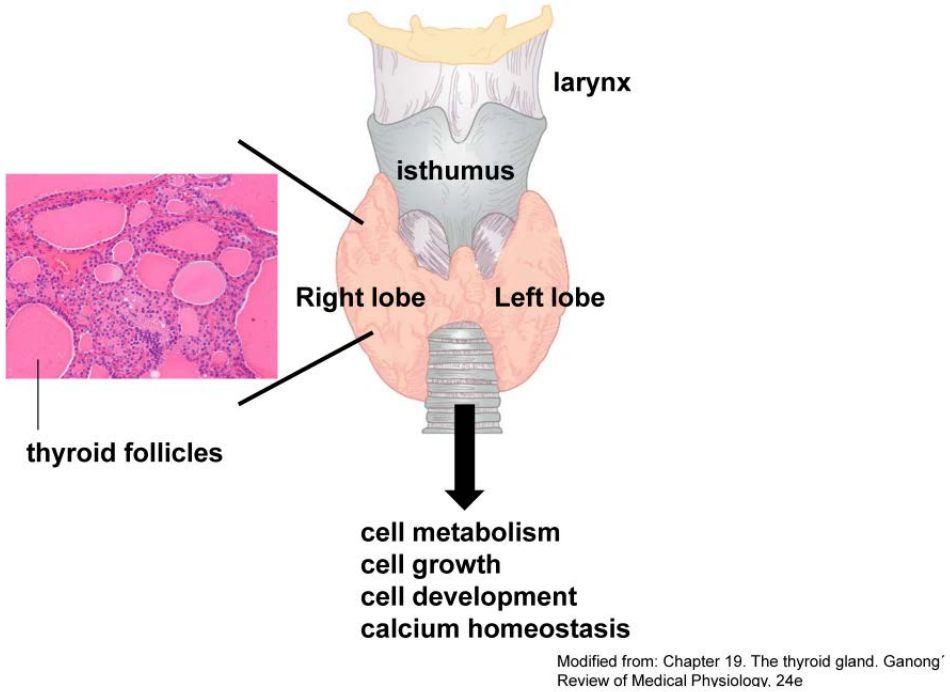

Figure 4. **Anatomy of the thyroid gland and its physiological functions**.

The basic structural and functional component of the thyroid gland is termed follicle (**Figure 4**). The follicle is surrounded by a single layer of thyroid follicular epithelial cells in which hormone is synthesized and stored. The follicle content is termed colloid, containing mature thyroglobulin and thyroid hormone in the form of triiodothyronine (T3) and thyronine (T4), which are synthesized and secreted by the follicular cells. The follicular cells absorb iodide from the blood, and then release it to the colloid where it is oxidized to iodine and binds to proteins such as thyroglobulin. The protein complex is then reabsorbed and undergoes proteolysis in follicular cells. Finally, T4, but also some T3, is released to the blood stream. T4 is peripherally de-iodonized to T3, which is the active form of thyroid hormone. The synthesis and secretion of thyroid hormones is subject to negative feedback regulation of the hypothalamin-pituitary-thyroid axis. Briefly, thyrotropin-releasing hormone (TRH), produced by hypothalamus, binds to receptors on the pituitary and stimulates the release of thyroid stimulating hormone (TSH). TSH receptors (TSHR) on thyroid follicular cells initiate the synthesis of thyroid hormones. In contrast, high concentration of T3 will inhibit the release of TRH and TSH, preventing the further production of thyroid hormone [39].

The thyroid plays an essential role in cell metabolism, growth and development via binding of thyroid hormone to thyroid hormone receptors in the cell nuclei. Calcitonin, produced by parafollicular C-cells, regulates calcium homeostasis in the human body. Thyroid dysfunction will cause increased or decreased thyroid hormone levels, inducing either hyperthyroidism or hypothyroidism, respectively.

1.2.2 Thyroid tumors

Thyroid tumors are classified into two main categories: adenomas and carcinomas. Follicular thyroid adenoma is the only benign thyroid tumor. Carcinomas can be grouped according to

their cellular origin, degree of differentiation and histopathological features. Adenomas are common in all populations all over the world, especially in parts of Europe, North America and Australia. Thyroid carcinomas are less common. It accounts for approximately 1% of all malignancies registered, but comprises 80% of all endocrine malignancies.

1.2.2.1 Follicular thyroid adenoma (FTA) and atypical thyroid adenoma (AFTA)

FTA is a common, follicular cell-derived thyroid tumor. The incidence is higher in women than in men. It can be described as a solid, round or oval, homogeneous tumor which is surrounded by a thin capsule. Adenoma cells can form regular follicles or a trabecular pattern; however, the tumor capsule and vessels are not invaded by tumor cells. The cytological pre-operative examination by fine-needle aspiration (FNA) cannot safely distinguish FTA from carcinomas since mitoses and pleomorphic changes may be overlapping features between these two entities [40, 41]. Once the patient is diagnosed with a thyroid follicular tumor by FNA cytology, lobectomy should be performed to further establish the diagnosis [42]. Provided no signs of malignancy are found by the histopathological examination, lobectomy alone is sufficient as the final treatment. This histopathological examination therefore includes a complete tumor capsule without any tumor in-growth, and lack of vessel invasion.

Follicular adenomas with high cellularity, abnormal nuclei, unusual histologic features and suspicious malignant potential are referred to as atypical follicular thyroid adenoma (AFTA). This specific entity is regarded as having an uncertain malignant potential. Besides AFTA, many variants of FTA have been recognized [40].

1.2.2.2 Medullary thyroid carcinoma (MTC)

MTC, arising from the parafollicular calcitonin-producing C- cells, is a differentiated form of thyroid carcinoma that accounts for 5-8% of human thyroid cancers. The C-cells are located at the basal layer of the thyroid follicles, and account for 1% of all thyroid cells. During fetal development, C-cells immigrate from the neural crest; hence, MTC is referred to as a neuroendocrine tumor. Approximately 75% of the cases are sporadic and the remaining 25% are parts of the autosomal dominant multiple endocrine neoplasia type 2 (MEN 2) syndrome [43]. MEN 2A is characterized by MTC, pheochromocytoma and primary hyperparathyroidism; MEN 2B is characterized by MTC, pheochromocytoma, ganglioneuroma and other phenotypes [44, 45]. The vast majority of MEN 2 patients carry a constitutional mutation of the *RET* (Rearranged during Transfection) proto-oncogene, but *RET* mutations also play an important role in sporadic MTC development. MTC usually occurs in the middle of the thyroid lobe where C-cells are located. It is a solid carcinoma causing increased calcitonin levels in serum.

Histopathologically, MTC exhibits sheets, nests or trabecular spindles, round or polygonal cells and is surrounded by a vascular fibrous stroma. Most MTCs are strongly positive for calcitonin staining. MTC tends to metastasize to lymph nodes, especially the cervical lymph nodes at an early stage. Distant metastases are frequently found in liver, bone and lung [40].

The initial treatment for MTC is total thyroidectomy plus lymph node dissection in the neck [46]. The calcitonin level in serum is an important prognostic marker; increased calcitonin post-operatively indicates persistent diseases, and further treatment or surveillance could be indicated. Recurrence is seen in 5-12% of patients several years after the first operation [47]. Presence of a somatic *RET* mutation, age, gender and distant metastases are risk factors affecting the survival of MTC patients [48].

1.2.2.3 Follicular cell-derived human thyroid carcinoma

Thyroid carcinoma originating from follicular cells consists of two well differentiated types: follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC). The poorly differentiated thyroid carcinoma (PDTC) and undifferentiated, or anaplastic thyroid carcinoma (ATC) may be the result of a stepwise dedifferentiating process from PTC or FTC, but may also arise *de novo*.

1.2.2.3.1 FTC

FTC accounts for 15-20% of all malignant thyroid tumors and it is the malignant counterpart of FTA and AFTA. FTCs are differentiated solitary tumors that can be more or less encapsulated. They typically occur in women at their 50s but one third occur in men. The histopathological features of FTC are hyper-cellularity, differentiated follicular cells, invasion of blood vessel and capsule but without the specific characteristics of PTC [40]. Depending on the degree of invasiveness, FTC can be sub-classified as minimally invasive and wildly invasive type. The latter can cause disruption of the tumor capsule and invade surrounding thyroid tissue, thus leads to less favorable prognosis. Metastases are found in the bone, lung and liver, but are rare in the brain [49, 50].

Oxyphilic cell type (Hürthle cell type) carcinoma is a variant of FTC. It accounts for 3-4% of thyroid cancer, and appears as a solitary tumor with large nuclei and plenty of mitochondria in the cytoplasm [40, 51].

1.2.2.3.2 PTC

PTC is the most common type of thyroid cancer (80-85%) that frequently occurs in women (75%), typically around menopause. The development of PTC is influenced by environmental factors such as radiation exposure, especially in children. The incidence of PTC increased rapidly in young people after the Chornobyl nuclear plant accident in Ukraine [52]. PTCs are differentiated thyroid carcinomas that appear as firm nodules without regular and clear borders; invasion to surrounding thyroid tissue may be observed. The microscopic picture has characteristic features including hyper-cellularity, enlarged nuclear, ground-glass appearing nuclei, grooves in nuclear contours and psammoma bodies in the tumor cell stroma or in lymphatic spaces [40, 51]. Patients with PTC usually undergo total thyroidectomy followed by radioiodine ablation. The overall prognosis for PTC patients is excellent, and is better than for other types of thyroid cancer [51, 53].

There are several variants of PTC such as follicular-variant PTC, tall cell PTC as well as a few rare variants.

1.2.2.3.3 PDTC

PDTC originates from follicular cells, but its morphology and behavior suggest that it is an intermediate form between differentiated carcinoma and ATC. PDTCs are solitary large tumors with various histopathologic features, including insular, trabecular and solid patterns. Infiltration to surrounding tissues and vessels and distant metastases are frequently observed. The prognosis for PDTC patients is much less favorable than for PTC and FTC [40, 51].

1.2.2.3.4 ATC

ATC is a rare, highly malignant form of thyroid tumor characterized by undifferentiated cells. ATCs are large, often infiltrating surrounding tissues and disrupting the structure of the normal thyroid. At diagnosis, they have commonly metastasized to regional lymph nodes and other organs. Histopathologically, ATC exhibits spindle cells and giant cells with multiple nuclei. Mitoses and necrosis are often seen [40]. The prognosis for ATC is dismal, and most ATC patients die within six months after diagnosis. Radiation combined with chemotherapy is used as palliation to improve the outcome of surgery [54, 55].

1.2.3 Genetic alterations in thyroid tumor development

1.2.3.1 Molecular genetic background of MTC

The development of sporadic and MEN 2 associated MTC is strongly linked to activating mutations of the *RET* proto-oncogene, which was identified by *in vitro* transfection with T-cell lymphoma DNA. The *RET* gene is located in chromosomal region 10q11.2 and comprises 21 exons [56, 57]. The encoded receptor tyrosine-kinase functions as a membrane receptor for glial-derived neurotrophic factor family (GDNF) ligands. It is critical for the development of sympathetic, parasympathetic and enteric neurons, the kidneys, germ cells and thyroid C-cells [58]. The protein has three domains: the extracellular ligand-binding domain with cadherin-like repeats and a cysteine-rich region, the trans-membrane domain, and the tyrosine kinase domain. As a signaling receptor, it binds to the GDNF family via GDNF-family receptor-α (GFR-α), leading to phosphorylation of RET tyrosine residues [59]. The downstream cellular signaling initiated by GDNF ligands can activate RET via two different mechanisms: *cis*- and *trans*-activation; which in turn will activate different downstream kinases [58].

Most cases of MEN 2 are caused by a constitutional *RET* mutation. *RET* was identified as the predisposing gene for MEN 2 in the 1990s by genetic linkage analyses in affected families [60, 61]. Point mutations in exon10 and exon11 are the most common causes of MEN 2A; the majority of MEN 2B is caused by a mutation in exon 16 (Met 918 Thr) which has often occurred *de novo*, and a few cases are caused by mutations at other codons for example 883 and 806 [62-65]. Approximately 40% of sporadic MTC cases have a somatic *RET* mutation in exon 16, and the M918T mutation is the predominant type. All *RET* mutations in MTC

lead to gain-of-function of the RET protein, among which the M918T mutation induces multiple down-stream signaling changes. On the one hand, M918T mutation allows the tyrosine kinase to be stimulated without ligand binding; on the other hand, RET substrates can be modified by the M918T mutation [58]. The M918T mutation has been associated with a poor outcome in patients with MTC [66, 67]. Besides *RET* point mutations, a translocation involving *RET* intron 12 was revealed in one female patient, and the *RET* fusion has been identified as oncogenic driver in sporadic MTC *in vitro* [68].

RAS mutation is another oncogenic event in MTC, which was first reported in 2011 [69]. *RAS* genes, including *HRAS, KRAS* and *NRAS*, are the most commonly mutated proto-oncogenes in human cancers. They encode approximately 21 kDa RAS proteins, that play an important role in transducing signals from the cell surface [70]. Activating mutations of the *RAS* oncogene have been thoroughly described in different kinds of tumors, and have also been established in follicular cell-derived thyroid cancer. The presence of different *RAS* mutations and their clinical implications in sporadic MTC have been reported in previous studies [71, 72].

1.2.3.2 Genetic alterations in other types of thyroid tumors

The most common genetic aberrations in PTC are *BRAF* (v-Raf murine sarcoma viral oncogene homolog B1) mutations and different chromosomal rearrangements. The *BRAF* gene in chromosomal region 7q34 encodes a protein that belongs to the serine/threonine kinase family, which is known to play a role in cancer development. The V600E substitution at position 1799 is frequently detected in PTC, and it has been identified as a prognostic marker for PTC; in addition, the *BRAF* mutation is also present in ATC (25%) and PDTC (20-40%) [73].

Several chromosomal rearrangements including the *RET* gene have been identified in PTC, and are thus termed *RET/PTC.* The para-centric inversion of chromosome 10 leads to *RET* fusions with other genes and subsequent activation of RET signaling pathways; *RET/PTC* fusion oncogenes are important genetic events in PTC development [74].

RAS mutation is a common genetic event in FTC and ATC [51]. The occurrence of *RAS* mutation in FTA, FTC and ATC suggests a role for *RAS* in tumor progression [75]. It is also detected in PDTC and FVPDC [51, 76].

The paired box 8 *(PAX8)* - peroxisome proliferator activated receptor γ *(PPARγ)* fusion is the outcome of the translocation t (2; 3) (q13; p25). This gene fusion is frequently observed in FTC (60%) and is considered as a specific marker for FTC; however, it has also been reported in FTA [77, 78].

In addition, copy number gains of proto-oncogenes in the PI3K-AKT pathway, promoter mutation and promoter methylation of *TERT* [79] as well as mutation and promoter methylation of TSGs are also involved in thyroid tumor development [51].

1.3 TELOMERE AND TELOMERASE

The semi-conservative DNA replication is initiated in the 5´-to -3´ direction by the RNA primer and DNA polymerase, and continues until the linear end of the chromosome in eukaryotic cells. The 5´gap in a newly synthesized daughter chromosome cannot be filled; therefore, chromosome ends become shorter after each successful replication. Nevertheless, the entire genetic information in the chromosomes is replicated in normal eukaryotic cells and chromosomes remain stable without fusing end-to- end. The reason for this phenomenon is the tandemly repeated sequences called telomeres that are present at the chromosome ends. Telomeres thus serve as protective shields for the chromosomes in eukaryotes. Telomeres are synthesized by a ribonucleoprotein enzyme called telomerase, which can polymerize nucleotides into tandem repeats of telomeric DNA sequence at the end of each chromosome.

Telomeres and telomerase exhibit alterations in many diseases including cancer, which are of biological and clinical significance, especially in cancer. In this thesis, the telomere maintenance, TERT dysregulation, telomerase reactivation and their clinical implications in thyroid tumors were explored.

1.3.1 Telomere

1.3.1.1 Its discovery and structure

In the 1930s, Muller created the term "telomere", and suggested that it is a special structure at the end of chromosomes [80]. Then in the 1940s, Barbara McClintock reported that chromosomes fused to each other after losing the special structure at chromosome ends in corn [81-83]. This discovery together with her pioneering work on transposons in corn brought her the Nobel Prize in 1983. In 1978, Elizabeth Blackburn revealed that TTGGGG sequences are tandemly repeated at chromosome ends in *Tetrahymena thermophile* [84].

Telomere, a complex of DNA and protein, is conserved on telomeric DNA in different eukaryotes. The telomere sequences consist of a double stranded DNA repeat sequence (TTAGGG in human), which are short and rich in G-residues on the 5´ to 3´ strand running towards the end of the chromosome [85]. This G–rich strand protrudes the duplex and forms a single stranded G-rich overhang termed G-tail [86]. In the S-phase of the cell cycle, the G-tail increases and the 3´ overhangs usually invade and insert into the double stranded telomere repeats to form a t-loop [87], and thus play a role in the telomere maintenance. The length of the telomeric repeat sequences varies between species, and also in relation to age and disease.

The proteins in the telomere complex can be divided into two categories: proteins directly binding to telomeric DNA and proteins interacting with binding proteins. In humans, the most important binding protein component is shelterin. Shelterin is a six protein complex consisting of TTAGGG repeat binding protein factor 1 (TRF1), TRF2, TIN2, Rap1, TPP1 and POT1. The duplex of telomere is bound with TRF1, TRF2 and POT1, which interacts with Rap1, TPP1 and TIN2. The shelterin complex forms a special structure with the

telomere, for capping and protecting the telomere, and maintaining the chromosome structure [88].

1.3.1.2 Telomere replication

The double stranded telomeric DNA is commonly replicated by the conventional DNA polymerase in each cell cycle. On the leading strand, the telomere can be replicated without loss of nucleotides. However, because of the cleavage of RNA primers, the telomere will lose a few nucleotides on the lagging strand. Telomerase can counteract the problem by polymerizing nucleotides into tandem repeats in the G-rich strand of the telomere. The G-tail at the blunt end of the telomere on the newly synthesized strand can be generated by C-strand degradation and telomerase-mediated elongation [89, 90].

1.3.1.3 Telomere function

Telomeres and their DNA-protein complex structures protect chromosome ends from end-to-end fusions, double-strand breaks and degradation. Besides maintaining the integrity of the chromosome, the telomere plays an essential role in life-span controlling of cells: the eroded telomere can induce permanent growth arrest (referred to as replicative senescence) and thus prevent cellular immortality [91, 92]. Telomere also participates into the process of chromosome positioning in the nucleus, heterochromatin formation and transcriptional repression in certain organisms [80].

1.3.1.4 Maintenance of telomere length

The telomere length is maintained by the balance between shortening and lengthening mechanisms, therefore the telomere structure and length are dynamic, not static in many organisms [93-97]. Several biological processes mediate the maintenance of telomere length that is affected in different diseases, including cancer. Telomerase-mediated elongation is the predominant pathway in humans; however additional mechanisms have been identified in other eukaryotes.

Alternative lengthening of telomeres (ALT) is a telomerase-independent mechanism observed in human tumors and immortalized cell lines. Recombination-mediated DNA replication has been considered as the main mechanism for ALT. It involves a rolling-like spreading of replication using circular telomeric DNA produced by homologous recombination. Telomeres on the sister chromatids can be used as template for elongation in the T-loop configuration [98, 99].

Cells with involvement of the ALT pathway are characterized by heterogeneous length of telomeres and nuclear DNA-protein complex which is referred to as ALT-associated promyelocytic leukemia (PML) protein nuclear bodies (APBs) [100]. The *PML* gene is identified in acute promyelocytic leukemia [101]. The PML protein has been proved to be involved in different biological processes in different studies, such as cell apoptosis pathways, gene transcription regulation, DNA repair pathways as well as the chromatin remodeling [102-106]. In addition, telomeric DNA and telomere-binding proteins as well as

proteins related to DNA replication and homologous recombination are also found in APBs[99].

Telomere binding proteins and DNA damage response proteins also play a role in telomere maintenance. In human, TRF1 can negatively regulate the telomere length by affecting telomerase. Rap1, binding to the telomere duplex, plays a role in telomere length regulation [107, 108].

1.3.2 Telomerase

1.3.2.1 Discovery and its expression in vivo

Telomerase was identified as a catalytic unit to extend telomeric sequences in Tetrahymena in 1985 by Elizabeth Blackburn and Carol Greider [109]. Soon after they discovered the ribonucleoprotein complex consisting of two distinct components, possessing the terminal transcriptase activity[110], and the RNA component was identified as the template for telomeric DNA repeats synthesis in the complex [111]. The discovery of telomerase brought Carol W. Greider, Elizabeth Blackburn and Jack W. Szostak the Nobel Prize in 2009.

In humans, the catalytic subunit for the synthesis of six nucleotides to the telomeric DNA is termed human telomerase reverse transcriptase (hTERT); the telomerase related RNA template is called hTR. The hTR is a 451 nucleotide non-coding RNA transcribed from *TERC*. The core region of hTR bearing nucleotide (5´-CUAACCCUAAC-3´) is complementary to the G-rich tail of the telomere [112]. The telomerase complex has other components besides TERT and the RNA template, for example dyskerin [113]. Several other proteins interact with telomerase which are involved in the trafficking, recruitment to telomere, telomerase biogenesis and degradation. However, they are not used as integral components of the holoenzyme and catalytic subunit [114].

Telomerase activity is detectable in 85% to 90% of human tumor tissues and cell lines. In addition, it is also present in early embryogenesis, testicular germ cells, stem cells and activated lymphocytes [115, 116]. In most normal somatic cells, telomerase is undetectable or present at very low levels; and the absence or a low level of telomerase activity cannot prevent progressive telomere erosion in normal cells. hTR is constitutively expressed in human cells, even in pre-crisis cells without detectable telomerase activity, whereas TERT is only expressed in cells with detectable telomerase activity. In addition, introduction of TERT into human normal fibroblasts induced telomerase activity, telomere elongation, sustained proliferation and reduced senescence, indicating that TERT is the rate-limiting subunit of the telomerase holoenzyme [112, 117-119].

1.3.2.2 Telomerase and its biological role in tumorigenesis

Normal cells have a finite lifespan because of the erosion of telomeres in each cell cycle. After undergoing a limited number of cell cycles, cells with short telomeres will reach their Hayflick limits [120], then finally enter into senescence or crisis. Hence, depletion of

telomeres is a barrier to cell immortality. Cancer cells need to acquire the capability of unlimited replication and division by maintaining the telomere length. Telomerase, repressed in normal cells, is functionally reactivated in the vast majority of human cancers and immortal cells [118, 121]. On the one hand, expression of telomerase alone is sufficient for the cell escaping from crisis and immortalization; one the other hand, expression of telomerase can cooperate with oncogenes or inactivated TSGs, permitting tumorigenesis [122].Taken together, telomerase activation has an important implication for diagnostic and therapeutic applications, and has been considered an important clinical biomarker for different types of tumors. An attractive strategy for anti-cancer therapy is the usage of telomerase inhibitors to prevent restoration of the telomeres and thus induce cell death [123-125].

In recent years, multiple studies have demonstrated that the function of telomerase is not only limited to telomere maintenance, but also to cell proliferation independent of telomere maintenance. Telomerase/TERT functions independent of telomere maintenance are observed in conditions where the telomerase enzymatic activity has been eliminated [126].The non-canonical roles of telomerase, and in particular its catalytic subunit TERT, have been revealed bearing functions related to the chromatin remodeling. For example, TERT serves as a co-factor of the β-catenin/LEF transcription factor complex, inducing amplification of the Wnt signaling pathway [127]. Other telomere lengthening-independent functions include involvement in RNA-dependent RNA polymerase function [128], and DNA-damage repair [129]. The contributions of these additional functions of telomerase to tumorigenesis remain to be fully elucidated.

In summary, the telomerase complex facilitates the capping of chromosome ends via telomeres, so telomere maintenance is the most prominent of a diverse series of functions to which TERT contributes. The function of telomerase in keeping chromosome integrity, promoting cell immortality, and telomere-independent functions confer the tumor development and progression.

1.4 REGULATION OF TELOMERASE IN HUMAN CANCER

Attributing to its role in telomere maintenance and tumor formation, telomerase has been recognized as the crucial factor in oncogenesis. TERT as rate-limiting catalytic protein is the core subunit of telomerase holoenzyme. Its expression is positively correlated with telomerase activity, and thus its regulation in human cells has been extensively explored. TERT/telomerase expression can be regulated at various levels, including genetic, transcriptional, post-transcriptional and epigenetic levels, and by post-translational modifications, which will be described briefly below.

1.4.1 Genetic aberrations of *TERT* in human cancer

The human *TERT* gene, consisting of 16 exons and 15 introns, presents as a single copy gene located in chromosomal region 5p15.33, and extends over 40kb [130-133]. As shown in **Figure 5**, the proximal *TERT* promoter was identified within the region from 300 bp

upstream of the translation starting site (ATG) to the 37 bp of exon 2 [132]. Within this region, several transcriptional binding sites are identified, such as E-box and GC- box (described in detail below).

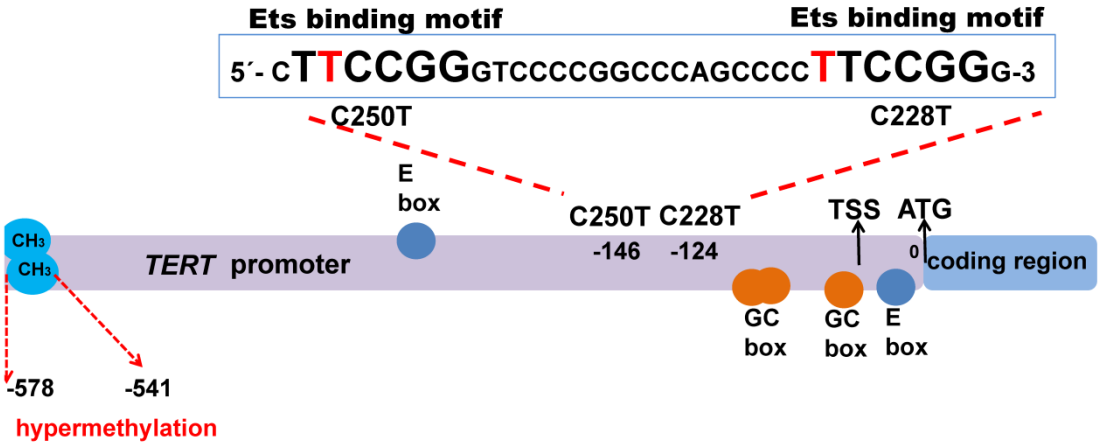

Figure 5. **Illustration of point mutations and methylation in the *TERT* promoter region**.

1.4.1.1 Copy number alterations

Copy number gains of the *TERT* gene locus are frequently observed in various human cancer tissues and cancer cell lines [134], which may due to the increased whole chromosome number or copy number gain of the TERT gene locus. Chromosome 5p, the location of *TERT* gene, is frequent gained in tumors [135]. In previous studies, the chromosome 5p was shown to be amplified in many types of tumors [136, 137]. Taken together, *TERT* copy number gains are common in primary tumor tissues and cancer cell lines, and associated with increased telomerase activity [138]. It is therefore considered as one of several reasons for telomerase activation.

1.4.1.2 TERT promoter mutation

Besides *TERT* copy number gains, point mutation of *TERT* is another genetic feature of cancer. The *TERT* promoter mutation was first reported in human melanoma in 2013 [139, 140]. As illustrated in **Figure 5**, two exclusive cytidine to thymidine (C > T) mutations locating at -124 and -146 bp from the ATG site are referred to as C228T and C250T mutation, respectively. These mutations create a new E26 transformation-specific (ETS) binding motif (5´- CCCCTTCCGGG-3´), stimulating *TERT* transcription [139, 141]. In addition to C228T and C250T, other mutations occur at low frequencies in this regulatory region, such as the CC > TT tandem mutations at -124/125 and -138/-139, the C > T mutation at -57. All these mutations promote the *TERT* transcription by creating new transcriptional factor binding sites [140].

These novel finding has inspired research on promoter mutation-induced TERT/telomerase up-regulation, which led to the discovery of frequent *TERT* mutations in other types of human cancers and immortal cell lines [79, 141-149]. Although mutation frequencies vary

among cancer types, endocrine tumors, hepatocellular cancers, renal pelvic cancers, gliomas, glioblastomas and skin cancers show high frequencies of *TERT* promoter mutations [146].

In some studies, the mutation was found to be associated with increased *TERT* expression; however, in other studies, no consensus was reached. The *TERT* promoter mutation has been shown to correlated with different clinical features, such as tumor progression and patient survival in various cancer types [150-153]; the importance of these findings is reinforced by the growing list of human cancers showing clinical associations with *TERT* promoter mutations.

C228T mutation is the major *TERT* promoter mutation in different types of thyroid tumors, but it is not present in normal thyroid tissues. The clinical implications of *TERT* promoter mutations have been explored in several studies. Undifferentiated thyroid cancer so called ATC displays the highest frequency of mutations (33-50%), PDTC shows a lower frequency than ATC but still higher than other types (29%); *TERT* promoter mutations in FTC (14-36%) is comparable to PTC (8-25%). *TERT* promoter mutations have also been detected in AFTA and in a single case of FTA [79, 143, 154-156]. The *TERT* promoter mutation in PTC is thus the second most common mutation in addition to the *BRAF* mutation which is regarded as the most common oncogenic event [146]. *TERT* promoter mutation was associated with tumor size, late tumor stage, and was found to be a predictor of metastasis and shorter patient survival in thyroid cancer. In addition, similar clinical implications were observed in renal carcinoma, glioblastoma and some other types of cancer [142, 157, 158]. In conclusion, all these findings indicate the *TERT* promoter mutation is an important genetic alteration in cancer which may serve as a useful biomarker in clinical practice.

1.4.2 Epigenetic changes of *TERT* in human cancer

The *TERT* promoter region lacks TATA or CAAT boxes but is rich in GC-nucleotides, indicating the epigenetic regulation by DNA methylation is involved in transcriptional regulation of *TERT* in normal and cancer cells [159, 160].

Promoter methylation is known to be associated with gene silencing, and inactivation of TSGs by hypermethylation plays a crucial role in tumorigenesis. However, the available literatures on the role of *TERT* promoter methylation in *TERT* regulation appear to be discrepant in different tumors and cell lines. Some findings showed methylation of the *TERT* promoter increased *TERT* expression [161], on the contrast, in others studies methylation of the promoter region was correlated with *TERT* silencing [162] [163]. In studies of *TERT* methylation analysis in breast, colon and lung cancer cell lines, *TERT* expression could be regulated via heterogeneous methylation status at specific regions. In the *TERT* promoter spanning a -150 bp to +150 bp region surrounding the transcriptional start site there is little or no methylation; however the -600 bp region upstream of the transcription start site is densely methylated, which is associated with *TERT* expression [164]. This study was based on immortalized cell lines; therefore more evidence should be obtained from studies of human

caner tissues in the future. Although the role of methylation for *TERT* expression differs from cancer to cancer, *de novo* methylation is acquired during tumorigenesis [162].

Histone modification mediated-chromatin remodeling has been demonstrated to involve into *TERT* expression regulation in previous studies [165, 166]. MicroRNAs, deregulated in cancer, were first revealed to be involved in *TERT* regulation in 2008 [167].

1.4.3 Dysregulation of *TERT* by transcription factors

The *TERT* promoter contains several transcription factor binding sites, and it can be regulated at multiple levels in different cellular contexts. So, transcriptional regulation is believed to be the dominant mechanisms for controlling TERT expression in human cancer. The transcription factors binding to the promoter are divided into subgroups according to their regulatory effects.

1.4.3.1 Positive regulators of TERT transcription

The *c-myc* oncogene plays a crucial role in a variety of human cancer via promoting cell growth, proliferation and immortalization [168]. It is well known that *TERT* is a direct target of c-Myc; in the core region of the *TERT* promoter, two E-boxes with the sequence of 5´-CACGTG-´3 are recognized and interact directly with c-Myc and Max heterodimers [169-171]. Over-expression of *TERT* induced by c-Myc is considered as an important mechanism of telomerase activation in many types of human cancer [172].

Sp1, universally expressed in normal cells, is a general transcription factor binding to GC boxes in promoters, enhancers as well as other regulatory regions. It stimulates a variety of genes in human [173]. Sp1 is a zinc-finger transcriptional factor, binding to five GC-boxes (5´-GGGCGG-3´) in the core promoter region of *TERT*, and co-operates with c-Myc to activate the transcription of *TERT* [174]. Overexpressed Sp1 increases the transcription of *TERT*, while mutation induced into the GC-box decreases the promoter activity [170].Besides cooperating with c-Myc, Sp1 can activate telomerase by interacting with MBD1-containing chromatin-associated factor (MCAF1) [175], high-mobility group A2 (HMGA2) [176] and nuclear factor of activated T cell (NFAT) in proliferating cells lines and cancer [177].

Besides these two crucial transcription factors, hypoxia-inducible factor-1(HIF-1) was observed to stimulate *TERT* transcription via binding to promoter regions in some types of cancers [178, 179], and activating enhancer-binding protein-2 (AP-2) binds to the *TERT* promoter to enhance *TERT* transcription in lung cancer [180]. In addition to transcription factors, oncoproteins may stimulate *TERT* transcription via different pathways: the human papillomaviruses (HPV) type-16 E6 oncoprotein binds to E6 associated protein (E6-AP) by repressing NFX1, promoting transcription of *TERT* [181]. RAS, RAF and growth factors stimulate *TERT* transcription via the ERK-MAPK pathway [182]. Estrogen binds to estrogen responsive elements in different promoter regions, inducing increased transcription [183]; it also up-regulates *TERT* at the post-transcriptional level [184].

1.4.3.2 Negative regulators of TERT transcription

Mad1 is a transcription repressor, and the Mad/Max/c-Myc network is essential in cellular transformation, differentiation and proliferation [169, 185]. Mad/Max heterodimers competitively binds to the E-box in the *TERT* promoter, repressing transcription of *TERT* [171, 186].

The transcription factor AP-1 is a heterodimeric protein of Jun and Fos family members. It can directly bind to the upstream positions -2000 and -378 of the *TERT* promoter region, and inhibit transcription [187].

As a tumor suppressor, p53 regulates many target genes involving in cell proliferation, differentiation, senescence and apoptosis. Since its function is inhibited in many types of cancer, p53 is essentially a tumor repressor in the context of tumorigenesis [188].*TERT* is inhibited by p53; p53 can prevent Sp1 binding to *TERT* promoter *in vitro*, attenuating the stimulatory role of Sp1 [189]. The p53-dependent repression also works via p53 binding to the core promoter region of *TERT*, with the cooperation of Sp1 [190].

Retinoblastoma protein (pRb) is a tumor suppressor which is inactivated in many types of cancer [191]. Hypophosphorylated pRb can down-regulate telomerase activity in cancer cell lines [192].

In addition, Wilms´ tumor 1 suppressor (WT1), PITX1, TGF-β and other transcriptional factors and TSGs prevent tumor formation via repression of telomerase [193-195].

1.4.4 Alternative splicing of *TERT* mRNA transcripts

Alternative splicing of RNA may be both tissue and disease specific, and will generate proteins with different functions [196, 197]. It is a common mechanism for gene expression regulation in human. The *TERT* gene is known to be alternatively spliced at different sites, producing multiple transcripts [196, 198-200]. Different *TERT* splicing variants are illustrated in **Figure 6**, and are described below.

The α-deletion transcript is generated via deletion of 36bp from exon 6 within *TERT* motif A; the deletion does not affect the protein translation, but it can negatively regulate telomerase activity and it is the dominant inhibitor [133, 201]. The β-deletion results from a deletion of 182 bp at exons 7 and 8, along with a 38 bp insertion at exon 4, producing premature terminations upstream of essential motifs [202, 203]. The γ- deletion transcript is generated via deleting exon 11and do not exhibit any function [200]. The remaining insertion transcripts occur at exons 11, 14 and exon 2. The insertion transcripts at exon 11 and 14 produce protein truncations at the N-terminal, thereby resulting in non-functional proteins [204].

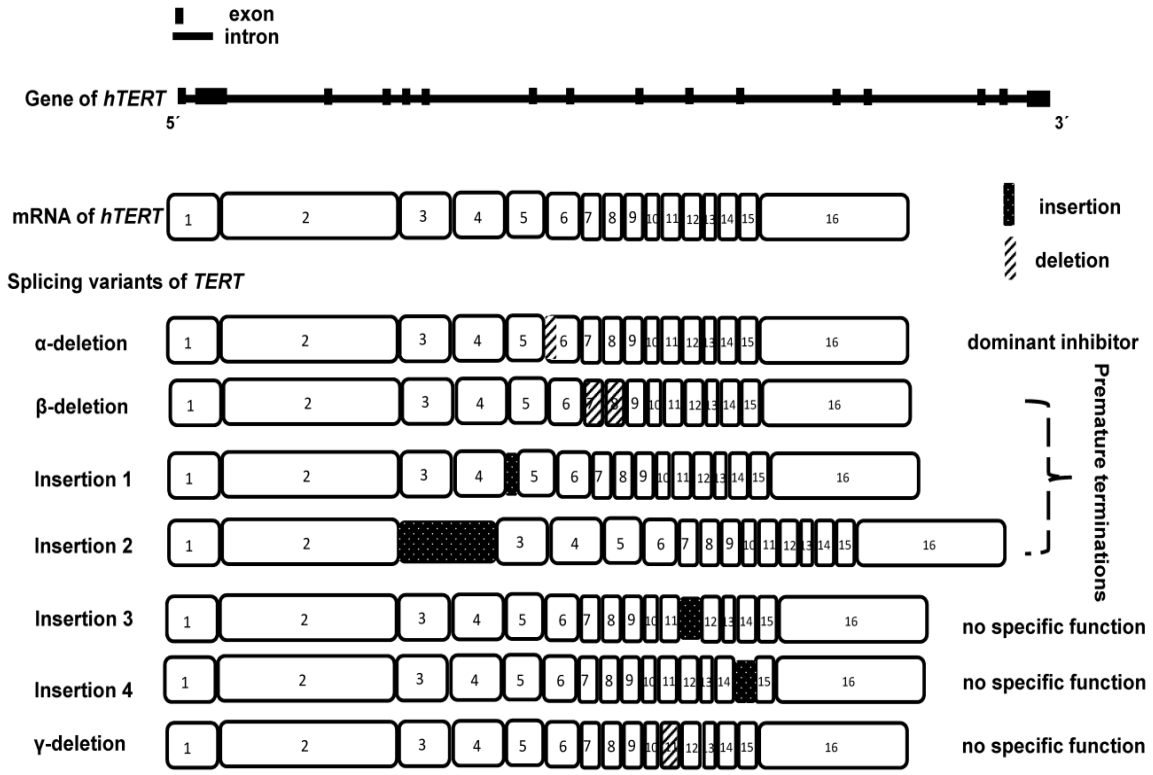

Figure 6. Different *TERT* mRNA transcripts generated by alternative splicing.

In previous studies of other cancers, telomerase activity was shown to depend on the existence of full-length *TERT* gene expression [199, 205, 206]. In conclusion, alternative splicing of RNA is an important post-transcriptional mechanism regulating telomerase activity.

1.4.5 Post-translational regulation of telomerase

Transcriptional regulation of the *TERT* gene is the dominant mechanism in controlling the telomerase activity. However, telomerase activity does not always corresponded to *TERT* expression, suggesting that post-translational modification of TERT protein plays a role in modulating telomerase activity. Phosphorylation of TERT is an important way of post-translational regulation by affecting different serine or tyrosine residues. Protein kinase B (PKB) and protein kinase C (PKC) increase telomerase activity via phosphorylating TERT catalytic enzyme [207-209]; whereas c-Abl decreases telomerase activity via phosphorylating TERT at tyrosine residues [210].

1.5 TELOMERASE IN THYROID TUMORS

The morphology of FTA and FTC may be similar, bringing a diagnostic challenge to histopathologists; in addition, the lack of predicting factors for the possibility of thyroid malignant transformation from adenoma makes treatment choices more difficult [211]. Telomerase activation, a promising marker for many types of cancer, has been explored in

different types of thyroid tumors [212]. Telomerase activity and *TERT* expression were detected at frequencies varying from 20% to 100% in PTC, and 30% to 100% in FTC and ATC [213]. According to previous studies, telomerase is a potential marker for distinguishing FTC from FTA, and it could also be used as useful diagnostic marker combined with routine cytology examination on FNA [211, 213, 214].

In addition, *TERT* expression and *TERT* promoter mutation have been associated with different aggressive clinical characteristics and shorter survival in thyroid cancer patients [79, 89, 154, 215-217]. The prognostic role of telomerase in thyroid cancer may contribute to the improvement of further therapy and patient follow-up.

In this thesis work, telomerase activation and its regulation in thyroid tumors were explored. The results indicate a prognostic value of telomerase in thyroid cancer.

2 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the telomerase activation and its clinical implications in thyroid tumors, and to further characterize the molecular genetic background of medullary thyroid carcinoma (MTC) in relation to telomerase activation.

The specific aims of the included papers were:

- To explore the *TERT* promoter mutation and its biological and clinical relevance in thyroid carcinomas (Paper I).

- To investigate the occurrence of *TERT* promoter mutation in thyroid follicular tumor development and to correlate it with clinical features (Paper II).

- To characterize mechanisms of telomere maintenance in MTC and the relationship to clinical features and survival (Paper III).

- To elucidate mechanisms of telomerase activation and the effects on protein expression profiles in MTC (Paper IV).

- To identify proteins regulated in relation to *RET* and *RAS* mutational status, and to investigate protein changes in response to RET signaling pathway inhibition in MTC (Paper V).

3 MATERIAL AND METHODS

Different cohorts of thyroid tumor samples and established human cancer cell lines were included in this thesis.

3.1 HUMAN THYROID TUMOR PATIENTS AND TISSUE SPECIMENS

3.1.1 Human medullary thyroid carcinoma (MTC) (Paper I, III, IV, V)

Forty-two MTC patients operated at the Karolinska University Hospital, Stockholm between 1986 and 2010 were included. The diagnosis was based on histopathological examinations following the criteria of the World Health Organization classification [40]. Forty-two fresh-frozen and 19 formalin fixed paraffin-embedded (FFPE) tumors were collected from Karolinska University Hospital Biobank. All fresh frozen tissues were snap-frozen in liquid nitrogen and then stored in -80 °C for further study after surgery. Tumor cell representativity was assessed by histopathological examinations of tumor sections from all tumor samples by a histopathologist at the Karolinska University Hospital. The clinical information for these 42 patients was retrieved from medical records and follow-up was done regularly with basal serum calcitonin measurement and clinical examinations.

3.1.2 Thyroid follicular cell-derived thyroid tumors (Paper I and Paper II)

Fresh frozen samples from 52 FTCs, 51 PTCs and 20 ATCs were obtained from the Karolinska University Hospital Bio bank. In addition, 58 FTAs and 18 AFTAs operated between 1986 and 2004 were collected after surgery.

3.1.3 Post-Chornobyl PTC (Paper I)

A cohort of 51 patients operated for PTC in Kyiv City Teaching Endocrinology Center, Ukraine was studied for comparison in Paper I. All these patients were exposed to radiation in connection to the Chornobyl nuclear plant accident in Ukraine in 1986.

3.1.4 Non-cancerous thyroid tissue (Paper II - Paper IV)

Twenty-four non-cancerous thyroid tissue samples from patients treated for other thyroid tumors than MTC (Study III, IV) and 20 specimens from non-tumorous thyroid glands (Study II) were used as references.

3.2 ESTABLISHED HUMAN CANCER CELL LINES

3.2.1 ATC cell lines (Paper I)

The human ATC cell lines U-hth7, U-hth74, U-hth104 and U-hth112, C634W and SW1736 were obtained from Uppsala University Hospital, Uppsala, Sweden; ARO and KAT-4 were purchased from ATCC. The ATC cell lines were characterized in previous studies in our group [218, 219].

3.2.2 MTC cell lines (Paper I and Paper V)

The human MTC cell line TT carrying a *RET* mutation in exon 11 was purchased from ATCC (LGC Standards GmbH, Germany). The MTC cell line MZ-CRC-1 harbouring a M918T *RET* mutation in exon 16 was obtained from Professor Bruce Robinson and Professor Stan Sidhu at University of Sydney, Australia.

3.2.3 Established non-thyroid cell lines (Paper III)

The human cervical cancer cell line HeLa (ATCC) and the human embryonic kidney cell line HEK 293 (Roche, Germany) were included as references.

All cell lines used in this thesis are listed in Table 1.

Table 1. Cells lines used in this thesis

Cell line name	Cell line origin	Source / Reference	Used in Paper
C643W	ATC	Uppsala University Hospital	I
SW1736	ATC	Uppsala University Hospital	I
U-hth 7	ATC	Uppsala University Hospital	I
U-hth 74	ATC	Uppsala University Hospital	I
U-hth 112	ATC	Uppsala University Hospital	I
U-hth 104	ATC	Uppsala University Hospital	I
ARO	ATC	ATCC	I
KAT-4	ATC	ATCC	I
MZ-CRC-1	MTC	University of Sydney, Australia	I and V
MTC-TT	MTC	ATCC	I and V
HeLa	Cervial Cancer	ATCC	II and III
HEK 293	Embryonic kidney	Roche, Germany	II and III

3.3 METHODS IN THIS THESIS WORK

In this thesis, different methodological approaches have been used for analyses on the DNA, RNA and protein levels. The experimental results were evaluated in combination with clinical characteristics of the patients.

GENETIC ANALYSIS OF *TERT, RET*, *RAS* AND *BRAF*

3.3.1 DNA extraction, RNA extraction and cDNA synthesize (Paper I – Paper V)

DNA was identified in the late 19th century, and its famous double helix structure was first described by James Watson and Francis Crick in 1953 and later awarded with the Nobel Prize in 1962. The heritable biological information carried by the DNA molecule lies in the liner sequence of nucleotides. The basic unit called gene generates RNA by transcription which is then translated into protein.

Genomic DNA from frozen tissues and cancer cell lines were extracted with DNase® Blood & Tissue Kit (QIAGEN, Germany). All frozen tissues were processed by slicing and incubated at 56 °C over-night in lysis buffer with RNase A. DNA was isolated and the final concentration was assessed by a NanoDrop ND-100 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

Total RNA was extracted from tumor tissues and cancer cell lines with the mirVana miRNA Isolation Kit (Applied Biosystems) and then stored at -80 °C. The RNA was quantified and quality assessed with a NanoDrop ND-100 spectrophotometer. Complementary DNA (cDNA) was synthesized from RNA using random primer (N6) and M-MLV reverse transcriptase (Invitrogen).

3.3.2 Polymerase Chain Reaction (PCR) (Paper I - V)

PCR is a widely used molecular biological technology that has revolutionized genetic studies in both basic and clinical areas. It was developed by Kary Mullis in 1983 and for his discovery he was awarded the Nobel Prize in Chemistry in 1993. The method is based on thermal cycling including repeated steps of denaturation, annealing and extension, and thus allows rapid amplifications of specific DNA regions. The starting material could be genomic DNA or cDNA generated from isolated RNA depending on the purpose of the research.

3.3.2.1 Reverse transcriptase PCR (RT-PCR)

RT-PCR is commonly used in RNA expression studies. In this method the RNA from genes of interest is first converted into complementary cDNA by reverse transcriptase. Subsequently, the newly synthesized cDNA is amplified by PCR and the products are visualized by agarose gel electrophoresis.

To explore *TERT* gene expression in tissues and cell lines, cDNA was synthesized from total RNA and subsequently used as template to amplify the *TERT* gene and *β-actin* gene. In **Paper III**, RT-PCR was used to verify the correct PCR products and to prepare the template for nested PCR.

3.3.2.2 Nested primer PCR

As an effective way to increase the specificity of PCR, the nested PCR is divided into two rounds. In the first round, the DNA template is amplified using a set of primers spanning the target areas; in the second round, the products from the initial amplification reaction are used as templates for a different set of primers which anneals closer to the target region. Therefore, non-specific fragments will be excluded, and the method can also be used for detecting differentially spliced RNA transcripts.

Nested PCR was performed in **Paper III** to detect the *TERT* mRNA splicing variants. In the first round the gene amplification, spanning the regions of all splice transcripts, was done by RT-PCR. The products were subsequently used as template for a second round of PCR reactions, in which a different set of primers was used.

3.3.2.3 Quantitative real-time PCR (qPCR)

qPCR is a rapid and efficient approach to simultaneously amplify specific nucleic acid sequences and measure the concentration of its products. There are two main methods to detect the amplifications. (1) double-stranded DNA is bonded by non-specific fluorescent dyes, which leads to the accumulation of fluorescence intensity corresponding to the increase of PCR products during the PCR reaction. SYBR Green is the commonly used dye binding double-stranded DNA. (2) A single-stranded DNA template, amplified by a set of primers and Taq polymerase, is hybridized with a sequence-specific oligonucleotides probe labeled with a fluorescent reporter at the 5´-end and a fluorescent quencher at the 3´-end. The close proximity of reporter and quencher inhibits the emittance of the fluorescent signals. However, the probe annealed to the template will be degraded by Taq polymerase along with the extension of a new DNA strand, permitting fluorescence from the fluorophore. Thus, the fluorescence detected by the real-time PCR machine is proportional to the increasing amount of products in each reaction. Taqman probe-based assay is the typical example of this method. Both SYBR green and Taqman probe assays were used in this thesis.

To quantify the nucleic acids amplified by qPCR, both relative and absolute quantification are applied. For absolute quantification the exact amount of target gene is given by comparison with the standard curve, while the relative amount of gene is obtained by comparison with an internal reference gene such as a stable expressed house-keeping gene.

In **Paper II** and **III,** the relative expression of *TERT* in thyroid tissues and cancer cell lines was determined by qRT-PCR using Taqman Gene Expression Assays (Applied Biosystems) and cDNA synthesized from total RNA as template. *18S rRNA* (Hs99999901_s1) was used as internal control and the relative gene expression was calculated based on the relative quantification method.

In **Paper IV**, *TERT* copy numbers were assessed by a qPCR-based method using Taqman® copy number assays (Applied Biosystems). Human *RNase P* gene which is known to exist in two copies in the human diploid genome, was chosen as internal control. The PCR reactions included purified genomic DNA from thyroid tissues, two primers, dNTPs, DNA polymerase, and two primers with dye-labeled probe: a FAM-dye labeled MGB probe for *TERT* and a VIC-labeled TAMRA probe for *RNase P*, respectively. The amplifications of *TERT* and *RNase P* were run in parallel in duplex qPCR reactions. The target copy numbers were calculated using a comparative quantification method ($\Delta\Delta C_T$): the C_T difference between the target and the reference genes was compared to the calibrator in two copies. An ABI 7900HT Real time PCR System (Applied Biosystems) was used and the copy numbers were predicated analyzed using the CopyCaller software (Applied Biosystems).

3.3.3 DNA sequencing

The DNA molecule is a large polymer with four different nitrogenous bases forming a linear backbone of alternating sugar and phosphate residues. In eukaryotes DNA is found in the chromosomes of the nucleus and in the mitochondria. The heritable biological information is

replicated at cell division resulting in the same order of nucleotides in the daughter cells as in the parental cells. The determination of the order of the DNA sequence in human and other species opened the gate to explore the genetic background of normal development and in disease.

Ever since the first gene of Bacteriophage MS2 was sequenced in 1972 by Walter Fires [220], there has been ongoing development of techniques for DNA sequencing. Today, fluorescence-based sequencing combined with automated analysis methods are widely used in basic and clinical research. In this thesis, Sanger sequencing and Pyrosequencing were used for various applications. The principles of these two methods are depicted in **Figure 7**, and **Figure 8**, and described below in details.

3.3.3.1 Sanger Sequencing

Frederick Sanger and his colleagues developed a method based on selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) in 1977 [221], which brought him the second Nobel Prize for Chemistry in his life. Over the past 30 years, Sanger sequencing has been widely used and developed; it still plays a crucial role for detection of genetic alterations in spite of the introduction of large-scaled, automated sequencing methods in recent years.

The method requires a single-stranded DNA template, a DNA polymerase and DNA primers, deoxynucleotides (dNTPs) and ddNTPs lacking of 3´-hydroxyl group. The failure of formation of phosphodiester bonds between two nucleotides will inhibit the DNA polymerase, thus the extension of the oligonucleotide chain will stop at the position of ddNTPs incorporation. The ddNTPs are labeled with different fluorescent dyes emitting light signal at specific wave-lengths, which facilitates the fragments generated by terminator ddNTPs visible. The resulting fragments with differentially labeled ddNTPs are separated and detected by capillary electrophoresis for the generation of chromatograms and sequence analysis.

In Paper **I, II** and **V**, point mutations in hot spot regions of the *TERT* promoter, *RET* and *RAS* were detected using Sanger sequencing. After PCR amplifications, the purified PCR products were precipitated with ethanol and EDTA, followed by electrophoresis in an ABI 3730 DNA analyzer machine. All steps were referring to the instructions in the BigDye Terminator V3.1 Cycle Sequencing Kit manual (Applied Biosystems).

The PCR products were sequenced in both directions and results were analyzed with the BioEdit Software and by visual inspection of the chromatograms.

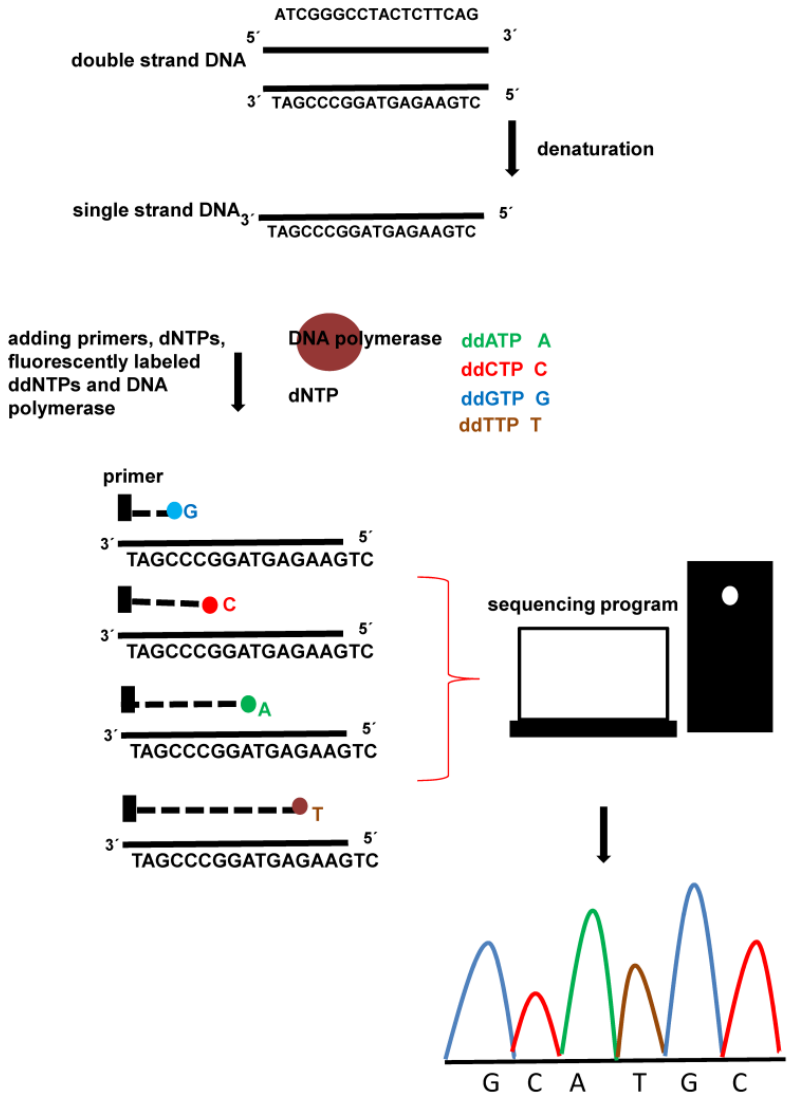

Figure 7. Illustration of the main principles of Sanger sequencing.

3.3.3.2 Pyrosequencing

Pyrosequencing was developed by Pål Nyren and Mostafa Ronaghi at Royal Institute of Technology, Stockholm in 1996, and has since then become one of the commonly used DNA sequencing methods. It is regarded as the standard technique for quantitative DNA methylation analysis and allows for the detection of single strand of DNA when synthesizing the complementary strand. The double-stranded DNA is treated with sodium bisulfite, which leads to the convention of un-methylated cytosine residues into uracil but leaves methylated cytosine unchanged. The bisulfite converted DNA is used for template of PCR amplification with biotinylated primers; the biotinylated amplified single strand DNA is then hybridized to sequencing primers and incubate with ATP sulfurylase, luciferase, apyrase and DNA polymerase, adenosine-5´-phophosulfate (APS) and luciferin. The incorporation of the first nucleotide initiates the reaction and then releases pyrophosphate (PPi), which could be used to synthesize ATP with APS by sulfurylase, and the synthesized ATP is the substrate of

luciferase. The conversion of oxyluciferin from luciferin by luciferase generates light signals proportional to the amount of synthesized ATP. The remaining unincorporated nucleotides and extra ATP will be removed by apyrase. The generated light will be captured by charged coupled devices (CCD) and visualized in the form of a Pyrogram. Thus the intensity of individual light signal corresponds to the number of nucleotides incorporated, and the ratio of methylated: unmethylated cytosine will represent the density of methylation at each CpG site.

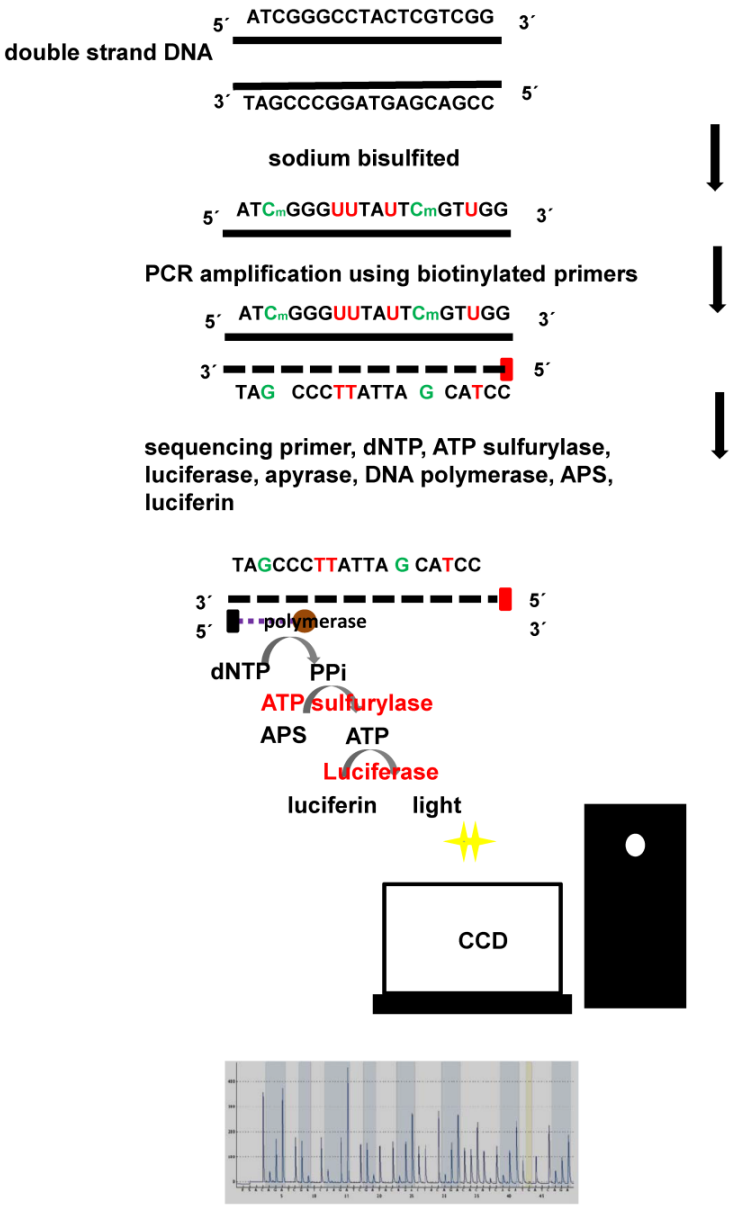

Figure 8. Schematic illustration of Pyrosequencing.

In **Paper IV**, the methylation density of the *TERT* promoter region in MTCs and normal tissues was identified by bisulfite Pyrosequencing. In addition, in **Paper I** the *BRAF* 1799 T>A mutation was determined by Pyrosequencing.

ASSESSMENT OF TELOMERE LENGTH

Various methods have been developed to detect telomere and measure the length of telomere [222-225]. The golden standard and the most reliable tool is Southern blot analysis of terminal restriction fragment (TRF). In addition, fluorescence in situ hybridization (FISH) and PCR based methods were developed to detect telomeres of individual chromosomes and determine the relative telomere length, respectively.

3.3.4 Southern blot and quantitative Real-time PCR

Southern blot analysis was invented by Edwin Southern in the mid-1970s and has been wildly used for detection of DNA sequence. The method involves separation of DNA fragments by electrophoresis, transfer and hybridization to specific probes. First, size-separated DNA fragments are transferred to a membrane after gel based electrophoresis. Second, the DNA fragments on the membrane are hybridized with a labeled probe. Third, the bound probe is detected by e.g. a chemiluminescent reagent.

In **Paper III** in this thesis, the distribution and absolute telomere length were detected using Southern blot analysis of TRF. Purified genomic DNA was cleaved with restriction enzymes that only cleave non-telomeric DNA but not telomeric DNA and sub-telomeric DNA. The products were separated by gel electrophoresis, transferred to a nylon membrane, and directly hybridized to a digoxigenin (DIG)-labeled probe which can only anneal to telomeric repeats. The DNA-probe complex was subsequently incubated with Anti-DIG-Alkaline Phosphatase. The final light signal representing the TRF was generated by metabolizing the chemiluminescent substrate by alkaline phosphatase. The TRF was compared with molecular weight markers and the calculation of mean telomere length was based on the density of chemiluminescent signals using the Image software.

In **Paper I, II,** and **III**, relative telomere length were assessed by a qPCR-based method (described above), including genomic DNA from frozen thyroid tissues, Platinum SYBR® Green qPCR SuperMix-UDG (Invitrogen, Germany) and a set of primers for *TEL* in each reaction. A melting curve was generated at the end of amplification to exclude non-specific binding between SYBR Green dye and primers. The single copy gene *HBG* was used as internal control. The ratio of C_t*TEL* and C_t *HBG* was calculated and taken as proportional to the mean relative telomere length.

All the PCR primers used in this thesis work are list in Table 2.

Table 2. Primers used in this thesis.

Target	Description	Forward and Reverse primer sequences (5´-3´)
Sequencing of RET		
RET exon 10	Forward	GCGCCCCAGGAGGCTTGAGTG
	Reverse	CGTGGTGGTCCCGGCCGCC
RET exon 11	Forward	CCTCTGCCGTGCCAAGCCTC
	Reverse	CACCGGAAGAGGAGTAGCTG
RET exon 15	Forward	GACTCGTGCTATTTTTCCTC
	Reverse	GCTTCCCAAGGACTGCCTGC
RET exon 16	Forward	AGGGATAGGGCCTGGGCTTC
	Reverse	TAACCTCCACCCCAAGAGAG
Sequencing of RAS		
HRAS1	Forward	ATGACGGAATATAAGCTGGT
	Reverse	CTCTATAGTGGGGTCGTATT
HRAS2	Forward	AGGTGGTCATTGATGGGGAG
	Reverse	AGGAAGCCCTCCCCGGTGCG
KRAS1	Forward	GGCCTGCTGAAAATGACTGAA
	Reverse	GGTCCTGCACCAGTAATATGC
KRAS2	Forward	CAGGATTCCTACAGGAAGCAAGTAG
	Reverse	CACAAAGAAAGCCCTCCCCA
NRAS1	Forward	ATGACTGAGTACAAACTGGT
	Reverse	CTCTATGGTGGGATCATATT
NRAS2	Forward	TCTTACAGAAAACAAGTGGT
	Reverse	GTAGAGGTTAATATCCGCAA
TERT expression		
TERT	Forward	CGGAAGAGTGTCTGGAGCAA
	Reverse	GGATGAAGCGGAGTCTGGA
β-actin	Forward	GCGGGAAATCGTGCGTGACAT
	Reverse	TGGCGTACAGGTCTTTGCGGATG
Sequencing of TERT		
TERT	Forward	CACCCGTCCTGCCCCTTCACCTT
TERT	Reverse	GGCTTCCCACGTGCGCAGCAGGA
Sequencing of BRAF		
BRAF	Forward	GCTTGCTCTGATAGGAAAATGAG
BRAF	Reverse	GTAACTCAGCAGCATCTCAGG
Telomere length		
Telomere	Tel 1b	CGGTTTGTTTGGGTTTGGGT-TTGGGTTT GGGTTTGGGTT
Telomere	Tel 2b	GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT
HBG3 (β-globin)		TGTGCTGGCCCATCACTTTG
HBG3 (β-globin)		ACCAGCCA-CCACTTTCTGATAGG

DETECTION OF ALT-ASSOCIATED PROMYELOCYTIC LEUKEMIA BODIES (APBS)

3.3.5 Fluorescence *in situ* hybridization (FISH)

FISH was developed in the 1980s to identify and localize nucleotide sequences. This cytogenetic tool utilizes complementary fluorescently labeled probes that bind to the sequences in specific chromosome region, and thus may detect and localize sequence targets in cells spreads or tissue samples. Quantitative FISH, a significant convenient and fast technique, has been used to detect and measure the telomere length for many years.

In **Paper III**, the telomeres were detected by FISH analysis in FFPE MTC tissue samples (described in details below).

3.3.6 Immunofluorescence

Similar to FISH, immunofluorescence is a method to detect and localize molecular markers in the cells. Different from the FISH technique, immunofluorescence utilizes a specific antibody to target the proteins of interest within a cell sample or a tissue section. The bound antibody is then visualized by a fluorescent dye and the protein localization may be observed using a fluorescent microscope.

In **Paper III**, APBs was detected by using immunofluorescence combined telomere FISH, to explore the interaction between PML and telomere. PML was firstly detected in FFPE slides using primary anti-PML mouse monoclonal antibody (Santa Cruz), which followed by Alexa fluor® 488 Florescent goat anti- mouse secondary antibody (Invitrogen). The Cy3- (5´-CCCTAA-3´)$_3$ PNA probe (Panagene, South Korea) was hybridized to the fixed slides for at least three hours, which was followed by staining with Tro-Pro®-3 iodide (Invitrogen) in mounting medium. The colocalization of the PML and telomere was visualized using a LSM 510 META confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

PROTEIN ANALYSIS OF TELOMERASE AND TELOMERASE RELATED PROTEINS

3.3.7 Assessment of telomerase activity

The telomeric repeat amplification protocol (TRAP) was developed by Kim et al in 1994 [121], and builds on the principle of PCR-based amplification of products generated by telomerase. As illustrated in **Figure 9**, telomerase may elongate the telomeric repeat sequences onto the non-telomeric oligonucleotide TS (5´- AATCCGTCGAGCAGAGTT-3´), followed by PCR amplification using upstream and downstream primers binding to the elongation products. The TS oligonucleotide is labeled by radioactive P or a flurophore, which will be detected and visualized using equipment for gel-based or chemiluminescent detection.

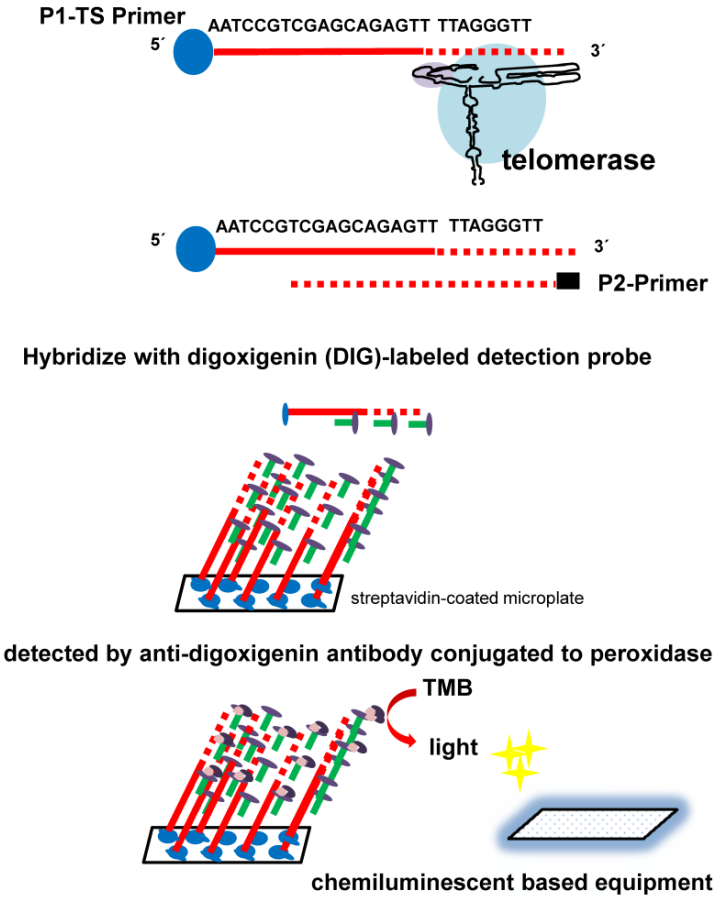

Figure 9. The principle of telomerase activity assessment.

In **Paper II** and **III**, the telomerase activity was assessed using the Telo TAGGGG Telomerase PCR ELISA kit (Roche, Germany). Protein extracts from thyroid tissues containing telomerase, which will elongate telomeric repeats to biotin-labeled synthetic TS primer. This step was followed by PCR amplification. The denatured single-stranded PCR product carrying biotin was hybridized to a digoxigenin-labeled probe. The assay involves an Elisa reaction by immobilizing the biotin-labeled PCR products onto the surface of a special plate, connecting the DIG-labeled probe and peroxidase labeled antibody against DIG. The final telomerase activity was determined by calculating the absorbance at different densities.

3.3.8 Mass spectrometry

Mass spectrometry (MS) is an increasingly used technology to identify proteins in biological substrates using a mass spectrometer. Three methodological variants are known: MALDI-TOF-MS, SELDI-TOF-MS and LC-MS/MS. In this thesis work, LC-MC/MS was used to identify proteins expressed in MTCs.

Proteins were extracted from tumor tissue samples followed by alkylation with iodoacetamide, digestion with trypsin, and iTRAQ labeling.

Protein samples were then separated on IPG strips with narrow (pH 3.7 – 4.9) and ultra-narrow range (pH 4.0 – 4.2), and eluted into 72 fractions per strip. At reversed phase LC-MS/MS peptides were fragmented to obtain the amino acid sequences, and peptides were identified by matching to a database with theoretical spectra from all human proteins.

In **paper IV** and **V**, the protein expression profiling of MTC samples was performed by HiRIEF-LC-MS/MS.

FUNCTIONAL STUDY *IN VITRO*

3.3.9 Assessment of cell viability

Cell viability is a very important biological feature of normal cells as well as cancer cells. Nowadays, several commercial assays are available for cell viability measurement. In metabolic activity measurements, the absorption of the dye solution is determined by a spectrophotometer. An increasing activity of the enzyme lactate dehydrogenase during cell proliferation will decrease tetrazolium salts in the surrounding environment of metabolic cells, which subsequently forms a formazan dye leading to color change of the media. Another commonly used protocol is to detect antigens associated with cell proliferation, such as Ki-67 using the MIB1 antibody.

In **Paper III** and **V**, the proliferation of MTCs was assessed by Ki-67 immunohistochemistry, and the cell viability of vandetanib-treated MTC cell lines was determined by the method based on metabolic activity.

4 RESULTS AND DISCUSSIONS

4.1 *TERT* PROMOTER MUTATION AND ITS CLINICAL IMPLICATIONS IN HUMAN THYROID TUMORS (PAPERS I AND II)

In 2013, constitutional and somatic mutations in the *TERT* promoter region, generating consensus binding motifs for ETS/TCF, were reported in melanoma by two different research groups [139, 140]. Since telomerase reactivation occurs in thyroid cancer, we speculated that *TERT* promoter mutation might be one potential underlying mechanism. In this thesis work, *TERT* promoter mutations were screened for in thyroid tumors and compared with clinical characters and patient outcomes in the Swedish and Ukraine cohorts.

4.1.1 *TERT* promoter mutation in human thyroid cancer

4.1.1.1 The TERT promoter mutations in FTC, PTC and ATC

A panel of follicular cell-originated thyroid carcinomas including 51 PTC, 36 FTC and 20 ATC in the Swedish cohort were screened for *TERT* promoter mutation at the hot spot positions chr5: 1,295,228 (C228T) and chr5: 1,295,250 (C250T) using Sanger sequencing. Totally, 31 tumors (29%) harbored one of these two mutations, of which C228T was found to be the dominant form (87%) compared to C250T. In conclusion, 12/51 PTC (24%), 7/36 FTC (19%) and 8/20 ATC (40%) exhibited the C228T mutation, while only one PTC, one FTC and two ATCs harbored the C250T mutation. No mutations were detected in the Ukraine cohort of 51 PTCs.

In addition, eight ATC cell lines were examined for *TERT* promoter mutation. The sequencing results revealed that the mutation occurred in 6/8 ATC cell lines. Similar to the tumor samples, the C228T mutation was more frequent than C250T which was only detected in one cell line.

4.1.1.2 TERT promoter mutation status in MTCs and two MTC cell lines

Thirty-seven sporadic MTCs and two MTC cell lines were screened for *TERT* promoter mutation at the sites 228 and 250. However, neither MTC tissues nor cell lines exhibited the *TERT* promoter mutation.

4.1.2 *TERT* promoter mutations in human thyroid adenomas

To further clarify the occurrence of *TERT* promoter mutations in thyroid tumor development, we included 58 patients diagnosed as FTA and 18 patients with AFTA in Paper II. The C228T mutation was identified in 1/58 FTA (2%) and 3/18 AFTA (17%). A panel of 20 non-tumorous thyroid tissues was screened as controls, and as expected, no mutation was found.

4.1.3 *TERT* promoter mutation is positively correlated with *TERT* expression in thyroid tumors

Constitutional and somatic *TERT* promoter mutations could create a new Ets/TCF transcriptional factor binding motif, which increases the *TERT* transcription level in melanoma and other types of cancers [226]. In this thesis, *TERT* expression and telomerase activity were assessed in thyroid adenoma tissues with different *TERT* promoter mutation status.

All the three AFTAs and one FTA with the C228T mutation showed detectable telomerase activity. One of seven FTAs without mutation expressed trace amounts of *TERT* transcripts, and 2/11 AFTAs without mutation exhibited *TERT* expression while another two exhibited trace levels of *TERT* mRNA expression. The *TERT* expression was found to be associated with telomerase activity in these tumors, and the *TERT* expression level was significantly increased in mutation positive as compared to wild-type tumors (Fisher´s exact test, $P = 0.008$).

4.1.4 The shorter telomere and age- dependent *TERT* promoter mutation in thyroid tumors

All 13 PTC patients and 7/8 FTC patients with *TERT* promoter mutation were older than 45. In the group of PTC > 45 years, there was a significant difference in age between mutated and wild-type cases ($P < 0.0001$). Furthermore, analysis of a second cohort of young patients who had been exposed to radioactivity from the Chornobyl accident, revealed no *TERT* promoter mutation. For FTC, the average age of patients in the mutation-positive group was higher than in the wild-type group ($P = 0.05$).

These observations suggested that the *TERT* mutation is age-dependent in PTC. Shorter telomeres and telomerase reactivation are important characteristics of many types of tumors. It is also well known that: (1) Reactivation of telomerase is triggered by telomere erosion; and (2) *TERT* expression is increased by creating new binding sites for transcriptional factors on the mutation sites. To further explore the background of the observed association, telomere lengths were determined in relation to *TERT* promoter mutation status.

First of all, we observed that the telomere length was not correlated with age in PTC patients (correlation coefficient: -0.22; $P = 0.127$, Pearson correlation).The mean telomere length was shorter in *TERT* promoter mutation positive PTCs compared to the mutation negative group (0.873 ± 0.45 and 1.569 ± 0.627; $P < 0.0001$). Second, for the subgroup of PTC cases > 45 years, the mean telomere lengths was shorter in cases with *TERT* mutation compared to the wild-type group (0.873 ± 0.45 and 1.451 ± 0.724; $P = 0.028$). Importantly, the *TERT* promoter mutation was found to be dependent on shorter telomere in PTC.

In FTA and AFTA, all cases with *TERT* mutation were > 45 years, but no significant correlation was observed between age and *TERT* promoter mutation. The cases with a C228T mutation had shorter telomeres vs. the wild-type group (3.97 ± 2.17 vs. 5.32 ± 2.95; $P > 0.05$).

4.1.5 Association of *TERT* promoter mutation with other common gene mutations in thyroid tumors

4.1.5.1 BRAF mutation

The *BRAF* mutation V600E is a common genetic alteration in PTC, and this mutation has been well studied *in vitro* and *in vivo*, and identified as an oncogenic event for PTC [51]. Given the high frequency of *TERT* promoter mutation (27%) and *BRAF* V600E (32/51, 63%) in PTC, we explored the possible relationship between the two alterations, however, no association was observed between these two genetic events.

4.1.5.2 RAS mutation

RAS mutations are highly prevalent in thyroid tumors. In FTA and AFTA activating mutations of three *RAS* genes (*HRAS*, *NRAS* and *KRAS*) have been reported in previous studies [227]. In our Swedish cohort of 18 AFTA and 58 FTA, neither *HRAS* nor *KRAS* mutation was detected. Five cases carried an *NRAS* mutation at codon 61 including two AFTA and one FTA with *TERT* promoter mutation. The conclusion was that patients with an *NRAS* mutation were prone to harbor *TERT* promoter mutation.

4.1.6 Clinical implications of *TERT* promoter mutations in thyroid tumors

4.1.6.1 Association of TERT promoter mutation with the progressive disease and poor clinical outcome in PTC

The *TERT* promoter mutation was significantly associated with cancer metastases (*P = 0.028*), as well as with disease survival (overall survival and disease-related survival) of PTC patients. The *TERT* promoter mutation independently predicted the disease-related survival in PTC.

4.1.6.2 Association of TERT promoter mutation with disease outcomes in FTC

The FTC patients with *TERT* promoter mutation had a shorter overall survival and disease-related survival (*P = 0.047* and *P = 0.025*, respectively). Similarly, the patient group carrying *TERT* promoter mutation stratified by age > 45 years tended to have shorter overall and disease disease-related survival (*P = 0.192* and *P = 0.099*, respectively). In the multivariate model, the presence of *TERT* promoter mutation significantly increased disease-related death risk (*P = 0.033*).

4.1.6.3 TERT promoter mutations are prone to occur in thyroid tumor with aggressive clinical behavior

The prevalence of *TERT* promoter mutation was 50%, 27%, 22%, 17% and 2% in ATC, PTC, FTC, AFTA and FTA, respectively; hence the mutation frequency paralleled with the aggressiveness of the disease. In agreement with this observation, *TERT* mutations were rare in FTA. The only FTA patient carrying a C228T mutation developed disease recurrence and subsequently died from FTC, whereas, FTA patients without mutation did not have detectable

recurrence, which might demonstrated that *TERT* promoter mutation played an important role in thyroid malignant transformation.

In summary, we identified *TERT* promoter mutation with different incidences in follicular thyroid cell-derived carcinomas (PTC, FTC and ATC), as well as in follicular thyroid adenomas (AFTA and FTA). Our results revealed *TERT* promoter mutation in thyroid carcinoma, with the characteristic of age and shorter telomere-dependent, could predict disease progression and survival. The study of *TERT* promoter mutation in AFTA and FTA demonstrated the presence of potential oncogenic alterations in thyroid pre-malignance.

4.2 TELOMERASE REACTIVATION AND TELOMERE MAINTENANCE IN MTC (PAPER III)

The involvement of telomerase in tumor development and its role in diagnosis and prognosis of many types of tumors including thyroid follicular cell-derived tumor has been investigated in previous studies [79, 125, 211], however, the role and clinical implications were less studied. In this thesis, the telomerase activation status in MTC and its clinical significance were explored, in addition to biological mechanisms involved in telomere maintenance in MTC.

4.2.1 Reactivation of telomerase in a subset of MTCs

Forty-two MTCs including 39 sporadic MTC and three MEN 2 cases were included in this study. The results revealed that 21/39 (54%) sporadic MTCs displayed *TERT* mRNA expression and corresponding telomerase activity, while the remaining 18 sporadic MTCs, three MEN 2 cases and normal thyroid samples showed no detectable *TERT* expression or telomerase activity. In telomerase-positive MTCs, the telomerase activity strongly correlated with *TERT* mRNA expression (r = 0.967; $P = 0.01$). In conclusion, reactivation of telomerase was identified in approximately half of MTCs.

4.2.2 Association of telomerase activation with clinical characteristics of sporadic MTCs

In the 39 sporadic MTCs, the telomerase reactivation was detected in 12/15 male and in 9/24 female patients, demonstrating that telomerase activation is more frequent in men than in women ($P = 0.02$). Histopathological and clinical parameters such as tumor stage, metastases, lymph node invasion have been used as predictive markers of MTC. Hence, in this study, we compared those parameters between the telomerase-positive and negative groups. All patients with telomerase activation were in tumor stage III and IV, while only half of cases had stage III and IV in telomerase activation negative group. Telomerase activation was associated with more advanced stage at diagnosis ($P < 0.0001$), with MIB-1 proliferation index (r = 0.683; $P = 0.01$) and with larger tumor size ($P = 0.027$).

4.2.3 Telomerase activation may predict the clinical outcome in MTC

Follow-up showed that among the 21 telomerase-positive cases, 13 died and the remaining eight were alive with spread diseases. In contrast, all telomerase-negative patients were alive at the end of follow-up except one who died of lymphoma. Patients free of disease displayed the lowest telomerase activity, while the cases who died of disease showed the highest telomerase activity. Meantime, significant differences in *TERT* expression and telomerase activity were observed between cases free of disease and patients with persistent disease, and patients with telomerase activation had a shorter survival compared to the telomerase-negative group. The multivariate analysis showed that the telomerase activation was an independent marker for shorter survival in MTC.

In conclusion, the telomerase activation was associated with poor clinical outcome and aggressive clinical phenotypes.

4.2.4 Telomere maintenance in MTC

As the essential structure at the end of chromosome, the telomere can prevent genome instability and preserve the chromosome integrity. Telomere DNA consists of tandemly repeated sequences, which are eroded gradually along with each cell division. The critically short telomere activates the DNA damage response signaling, and triggers the cellular senescence or apoptosis in normal cells, thereby avoiding the immortalization of cells. Therefore, the capabilities of stabilizing telomeres and thus escape from senescence and sustaining proliferation are essential for cancer cells. In this study, we investigated the mechanisms of telomere maintenance in MTC.

4.2.4.1 Detection of shorter telomere lengths in MTCs

Relative telomere lengths were determined by using real-time based PCR in 42 MTCs and 24 normal thyroid tissues. The MTCs displayed shorter telomeres as compared to normal thyroid tissues (*P = 0.0001*). The telomere length was also shorter in the telomerase-positive cases as compared to telomerase-negative MTCs.

4.2.4.2 Telomerase activation and the ALT pathway are two main mechanisms for telomere maintenance in MTC

Telomerase is activated in more than 90% of malignancies, and thus becomes the predominant mechanism for telomere maintenance in cancer. In MTC, shorter telomere and telomerase activation were identified in a subset of MTCs. In addition, ALT-based chromosome recombination was detected in some MTCs. Two key features of ALT (heterogeneity of telomere and presence of APBs) were observed in most telomerase-negative cases.

In summary, two mechanisms for telomere maintenance were identified in MTC. As the dominant mechanism, telomerase could be developed as a prognostic marker for MTCs.

4.3 MECHANISMS OF TELOMERASE REACTIVATION IN MTC (PAPERS III AND IV)

In the previous study, telomerase activation and its prognostic implications were revealed in MTC; however, the mechanisms underlying the activation are not well studied. The telomerase ribonucleoprotein complex, consisting of reverse transcriptase, RNA template and several proteins, may be regulated by different factors. In MTC, we investigated the dysregulation of telomerase using genetic and epigenetic methods and protein expression profiling.

TERT, the rate-limiting component of the enzyme, is well studied in many types of cancer. In MTC, we demonstrated that *TERT* expression was regulated via different mechanisms.

4.3.1 *TERT* promoter mutation in MTCs (Paper I)

Thirty-seven MTCs in our panel of 42 cases were screened for *TERT* promoter mutation using Sanger sequencing, however, neither C228T nor C250T was observed in these MTCs. The result is different from the situation in follicular-cell derived thyroid carcinomas where the *TERT* promoter mutation was established as a mechanism of TERT up-regulation and telomerase reactivation. Our results suggest that telomerase reactivation in MTC is not caused by *TERT* promoter mutation.

4.3.2 *TERT* copy number changes in MTCs (Paper IV)

Three copies of the *TERT* gene locus were detected in 5/39 sporadic MTCs (13%). The remaining 34 sporadic MTCs and three MEN2 cases revealed two copies of the *TERT* gene locus similar to the 10 normal thyroid samples analyzed for comparison. All five MTCs with copy number gains displayed *TERT* expression and telomerase activity, three of which showed high levels of telomerase activity. These five cases all presented with late disease stage at the diagnosis. The results showed that *TERT* copy number gains occur in a subset of MTCs and indicate that this alteration is associated with *TERT* up-regulation and progressive disease in MTC.

4.3.3 *TERT* promoter methylation in MTCs (Paper IV)

4.3.3.1 TERT promoter hypermethylation in MTCs

TERT promoter methylation was quantified at eight CpG sites located 600 bp upstream of the translation starting site by Pyrosequencing in MTCs and thyroid tissues. Hypermethylation (MetI >10%) was observed in 27 sporadic MTCs and three MEN2 cases; however, the normal thyroid sample exhibited low MetI only (below 10%). Taken together, the MTCs showed significantly increased *TERT* promoter methylation levels comparing to normal thyroids ($P < 0.0001$).

4.3.3.2 TERT promoter hypermethylation in telomerase reactivated MTCs

In our cohort, the *TERT* methylation level was found to be correlated with *TERT* mRNA expression ($r = 0.432$; $P = 0.006$) and inversely with the telomere length ($r = -0.343$; $P = 0.032$). MetIs were lower in telomerase-negative MTCs compared to telomerase-positive tumors ($P = 0.0001$).

4.3.3.3 Association between TERT MetI and clinical characteristics in MTCs

Comparison of MetIs with the tumor size, patient gender and tumor stage revealed significant associations to tumor size. Using the T-classification in the TNM system, higher MetIs were observed in tumors with extensive extra-thyroidal growth (T4) as compared to tumors classified as T1, T2 and T3 ($P<0.05$ in all comparisons).

RET and *RAS* mutations are common oncogenic events in MTCs, hence, the possible association between these mutations and *TERT* MetI was investigated. Higher MetIs were observed in *RAS* mutated cases ($P = 0.031$) as well as in *RAS* and/or *RET* mutated cases ($P = 0.002$) as compared to the respective wild-type cases.

4.3.3.4 TERT promoter methylation as a candidate prognostic marker in MTC

The survival was compared by log rank test for MTCs with high MetI above the median of 52% (for all cases studied) and MetI below the median level. The results were visualized in Kaplan-Meier plots which showed shorter overall survival ($P = 0.005$) and disease-related survival ($P = 0.007$) for MTC cases with high MetI. These observations suggested that *TERT* promoter methylation could be developed into an additional prognostic marker for MTC upon verification of the finding in independent tumor series.

4.3.4 *TERT* alternative splice variants in MTCs (Paper III)

In follicular cell-derived thyroid tumors, the *TERT* promoter mutation has been described and identified as one possible mechanism for telomerase activation [79, 143, 146, 155]. In addition to this, alternative splicing of *TERT* has been reported [206]. In thyroid and other tumors, the telomerase activity was found to depend on the expression of full-length *TERT* transcript [199, 205]. In our study, three of four *TERT* alternative splice variants were identified in 21 sporadic MTCs with telomerase activation. Seventeen among 21cases showed a combination of full-length transcript variants, α^- deletion and β^- deletion variants; one case only exhibited the full-length variant while the remaining six cases showed the β^- deletion transcripts only. None was detected to have γ^- deletion variant. Cases with the full-length transcript had shorter survival compared to cases where this transcript was not detected ($P = 0.04$). In conclusion, the *TERT* full-length transcript was associated with the clinical outcomes, implying its important role in telomerase functions in MTC, however, telomerase activity was not fully dependent of the full-length transcript.

4.3.5 Protein expression profiling based on telomerase positive and negative in MTCs (Paper IV)

Using HiRIEF-LC-MS/MS profiling of 14 MTCs a total of 4,321 proteins were identified and quantified, and subsequently analyzed in relation to *TERT* mRNA expression and telomerase activation. No obvious grouping of MTCs could be observed in the principal component analysis (PCA) model.

Totally, 240 of the 4,321 quantified proteins showed differential expression between telomerase-positive and negative MTCs ($P<0.05$). After further refinement of interesting proteins two enriched pathways were identified: "DNA double-strand break repair by non-homologous end joining" and "telomere extension by telomerase". *XRCC5* (Ku80) was present in both of two pathways. Expression of the *XRCC5* gene was found to be increased in MTCs as compared to normal thyroid tissues ($P = 0.007$), and it revealed a positive correlation to *TERT* promoter methylation ($r = 0.442$, $P = 0.007$). However, no significant difference in *XRCC5* expression was observed in relation to telomerase activation status.

In summary, telomerase activation in MTC could be attributable to multiple mechanisms. *TERT* promoter methylation at a specific region could predict disease outcomes. Dysregulation of proteins based on telomerase activation status in MTC need further clarification.

4.4 GENETIC BACKGROUND AND PROTEIN PROFILES IN RELATION TO *RET* AND *RAS* MUTATIONS IN MTCS (PAPER V)

Activating *RET* mutations are frequently detected at certain exons in patients with MTC, and *RET* has been established as a disease-related gene. In addition to this, somatic *RAS* mutations have been documented in thyroid tumors including MTCs. In this study, we screened for *RET* and *RAS* mutations in a Swedish MTC cohort, and investigated the relationship between these genetic events and clinical characteristics of the patients. By applying the recently developed HiRIEF-LC-MS/MS methodology for protein expression profiling, proteins related to genetic changes were explored in MTC tumor tissues and in two MTC cell lines that were treated with vandetanib.

4.4.1 *RET* and *RAS* mutational status in MTCs

Among 39 sporadic MTCs, somatic *RET* mutations were detected in 21/39 cases (54%) including the mutations M918T, C611S, C634Y, C634W and A883F. The three MEN 2A patients all harbored a constitutional mutation in the form of C634G, C634R and L790F, and in addition a somatic A883F mutation was detected in one case.

RAS mutation was detected in 7/41 MTCs (17%), which included the mutations G13R in *HRAS*, G12A in *KRAS*, Q61R in *KRAS* and G13R in *NRAS*. Six of seven *RAS* mutations occurred in *RET* wild-type cases, which was in agreement with the suggestion of mutual exclusivity between *RAS* and *RET* mutations.

4.4.2 Relationship between *RET* and *RAS* mutations with clinical features in MTCs

MTCs with a *RET* mutation were younger at diagnosis compared to wild-type cases ($P = 0.02$), and the M918T mutation tended to be associated with advanced tumor stage in MTCs ($P = 0.122$). However, there was no significant difference between *RET* mutated and wild-type MTC cases concerning patient outcome. No significant differences for age, gender, tumor size and survival regarding *RAS* mutation in MTCs were found.

Comparison of protein expression profiles between MTCs with different mutational status revealed quite limited sets of proteins that were differentially expressed between mutated and wild-type tumors. The corresponding pathway analysis of dysregulated proteins did not reveal significant enrichment for specific pathways. This suggested that MTCs that are wild-type for *RET* or *RAS* mutations carry oncogenic events that to a large extent involve similar molecular mechanisms.

4.4.3 Vandetanib decreases cell viability and induces protein expression changes in MTC cell lines

Two MTC cell lines with different *RET* mutations were treated with vandetanib for 72h, 96h until 7 days. Decreased cell viability was observed in both two cell lines. Protein expression profiles of two MTC cell lines after 2, 6 and 24 hours of treatment showed that 685 proteins

were regulated in response to vandetanib treatment. Nuclear protein 1 (NUPR1) and TP53 were identified as upstream activated regulators in both cell lines.

When comparing the proteins regulated by vandetanib treatment of MTC cell lines with those quantified in MTC tissues, an overlap of 110 proteins was identified, however this protein set could not separate *RET* mutated and *RET* wild-type cases at clustering. Six proteins were found to be common between the signature of RET regulated proteins and those regulated by vandetanib. Among them, nerve growth factor inducible (VGF) was decreased in MTC cell lines after treatment, and showed lower expression in MTCs with M918T *RET* mutation. The function of these proteins needed to be clarified further.

In summary, dominant *RET* mutations were frequently detected in MTC, in addition, *RAS* mutations occurred at a lower frequency in MTCs. Vandetanib treatment decreased MTC cells viability *in vitro,* and resulted in differential protein regulation.

5 CONCLUDING REMARKS

Telomerase/TERT, as a diagnostic and prognostic marker, has been reported in various tumor types. However, the clinical implication and underlying mechanism in thyroid tumors have not yet been determined. This thesis aims to explore telomerase activation in thyroid tumors, and then to further clarify the molecular background in relation to telomerase activation.

The general findings are summarized as follows:

1. *TERT* promoter mutations occurred in follicular thyroid cell-derived thyroid carcinomas with rather high prevalence. The mutation was identified in follicular thyroid cell-originated adenomas. *TERT* promoter mutation could predict thyroid cancer progression and adverse clinical outcomes.

2. Telomerase was activated in approximately half of MTCs, and its activation could serve as a prognostic marker for MTCs.

3. *TERT* was regulated at the genetic, epigenetic, transcriptional and translational levels in MTCs. *TERT* promoter methylation in MTCs could predict disease outcomes.

4. Besides telomerase activation, the ALT mechanism was observed in a subset of MTCs without telomerase activation.

5. *RET* and *RAS* mutations were detected in MTCs, and were associated with distinct sets of regulated proteins.

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